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The ultramicro determination of inorganic sulphate. By B. Spencer

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2. Reversal of respiratory decline in nutritional liver necrosis by intraportal administration of tocopherols and other substances. By J. Green, E. E. Edwin, J. Bunyan and A. T. Diplock

3. The relative activities of tocopherols and some other substances *in vivo* and *in vitro* against dialuric acid-induced haemolysis of erythrocytes. By J. Bunyan, J. Green, E. E. Edwin and A. T. Diplock

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Phosphoinositides:

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2. The inositol 1-phosphate structure in liver phosphatidylinositol. By J. N. Hawthorne, P. Kemp and R. B. Ellis

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Footnotes. These should be avoided in the text as far as possible.

The Autonomous Production of Individual Serum Proteins by Tissue in Culture

BY Y. M. ABDEL-SAMIE,* E. BRODA
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(*Received* 24 *July* 1959)

The production of individual serum proteins by individual kinds of tissue is difficult to prove or to disprove in experiments with intact animals or with surviving organs, since these contain a multitude of different kinds of tissue, often of overlapping activities and capable of mutual interaction. It is therefore preferable to investigate uniform tissue in isolation, i.e. in culture. Such tissue proliferates, in principle, indefinitely. Its biochemical potentialities presumably do not exceed those of the same kind of tissue *in vivo*, though they might be more restricted; thus the positive proof that a particular protein is synthesized by tissue in culture indicates that such synthesis may also proceed *in vivo*.

Intact animals or surviving organs are impregnated with fluids containing serum proteins, e.g. blood. It is therefore necessary when using them in experiments on protein synthesis to distinguish between pre-existing protein and newly synthesized protein. This can be done by the use of labelled amino acids, when only the newly formed protein will contain the label. Even in experiments with tissue cultivated in a medium originally free of serum proteins, however, the distinction between preformed and newly formed proteins is still needed in view of the protein contents of the cells themselves. For this reason, the use of isotopes is almost indispensable in this case as well. Moreover, the use of radioactive isotopes gives a method where the sensitivity of measurement is very high.

Three kinds of tissue were used for our experiments, namely mesenchyma tissue from chick embryos, epithelial-tumour HeLa tissue and a transformed human-liver-cell strain HLM. The tissue was incubated with nutrient medium containing [^{14}C]amino acid, and subsequently the soluble proteins of the tissue and medium were separated and fractionated by selective precipitation with ethanol. Electrophoresis in starch gel, followed by staining, was carried out as a test for the purity of the fractions. Finally, the radioactivity of the fractions separated with ethanol was measured; in one series (Table 3) the radioactivity of the eluates from the electrophoresis gels of such fractions was measured. The purity of the preparation of serum albumin has also been tested radioimmunochemically.

Various control experiments were needed to make sure that the results were not falsified by adsorption or other processes simulating genuine incorporation of amino acid. The most striking result of our work is the proof that both chick-mesenchyma tissue and HeLa-tumour tissue make serum albumin.

A preliminary report of some of our results has been given (Abdel-Samie, Broda, Kellner & Zischka, 1959).

EXPERIMENTAL

Materials

Radioactive amino acids. The makers of the radioactive amino acids (The Radiochemical Centre, Amersham, Bucks.) state that the specific activities of generally labelled L-[^{14}C]tyrosine hydrochloride and DL-[1-^{14}C]leucine were 54·3 and 41·6 μc/mg. respectively and that these amino acids were better than 99% radiochemically pure. They were used without further purification.

p-*Fluorophenylalanine.* This was supplied by L. Light and Co. Ltd.

Ethanol. A commercial grade of 95% ethanol was used without further purification, and the concentrations were determined by density measurement with a hydrometer at 25°.

Acetate buffers. Acetate buffers of known pH and ionic strength were prepared according to a nomogram (Boyd, 1945).

Serum-albumin antigens. The albumin for the immunochemical study was isolated by ethanolic fractionation and tested for its purity by electrophoresis in starch gel and staining (see below). Solutions (2%) of the albumin in 0·85% NaCl soln. were prepared, sterilized in the cold by filtration through a sintered-glass filter (Schott G5) under pressure, and kept ready for injection in 10 ml. vials in the refrigerator.

Rabbit antisera to serum albumin. Rabbit antisera against human-serum albumin (anti-HSA) and against chick-serum albumin (anti-CSA) were obtained by giving two intravenous injections of 5 ml. of 2% solution of

* On leave from the Clinical Pathology Department, Faculty of Medicine, Alexandria University, Alexandria, Egypt, U.A.R.

electrophoretically pure serum albumin, with an interval of 1 week, followed by one intra-abdominal injection of 10 ml. 1 week later. The sera obtained by bleeding 10 days after the last injection were purified by absorbing the anti-HSA with chick serum and the anti-CSA with human serum. The titres were determined by adding constant amounts of the antiserum to different dilutions of the respective antigens and finding the lowest dilution of antigen at which it still gave a precipitate with the antiserum.

Methods

Calibration of antisera. A calibration of the antiserum was performed afresh each time with the radioactive fraction to be used. When increasing amounts of the antigen are added to a given quantity of antiserum, the amount of precipitate passes through a maximum (Heidelberger & Kendall, 1935, 1937; Pappenheimer, 1940). Therefore different serial dilutions of the antigen were added to a constant volume of antiserum. The optimum ratio for precipitation was found either by determining, at each point, the nitrogen in the precipitate, or by means of the ring test. The nitrogen was estimated by treating the precipitate in the Kjeldahl apparatus, distilling the ammonia, adding Nessler's reagent to the distillate and measuring spectrophotometrically (Beckman DU) the absorption at 420 mμ. For the purpose of the ring test, an antigen layer was introduced in a microtube on top of the antiserum, and the white ring between the layers was observed visually after 15 min. The ring appears fastest and shows maximum intensity at the ratio optimum for precipitation.

In treatment with anti-HSA, a good precipitate was obtained in any case because of the presence of a large amount of (non-radioactive) human-serum albumin in the nutrient medium. However, to obtain good precipitates with anti-CSA, it was necessary to add inactive chick-serum albumin as a carrier, as the nutrient medium contained only a small amount of material from chick, i.e. the embryo extract. Therefore the addition of 5–10 mg. of inactive chick-serum albumin to the whole amount of radioactive albumin obtained by ethanolic fractionation from one to two roller tubes was necessary before determining the optimum ratio for precipitation.

Tissue cultures. The mesenchyma tissue ('fibroblasts') was obtained from legs of chick embryos 11–12 days old and was grown as monolayers in roller tubes at 37°, as described by Kellner & Stockinger (1957) and Abdel-Tawab, Broda & Kellner (1959), in a nutrient medium (1 ml./tube) consisting of 4% of chick-embryo extract, 40% of human ascites fluid and 56% of iso-osmotic buffered-salt solution (Gey, 1933).

Monolayers of HeLa cells were made in an analogous fashion from the HeLa tissue grown in these Laboratories for several years; HeLa tissue was originally derived from a human-cervix carcinoma (Gey, Coffman & Kubicek, 1952; Scherer, Syverton & Gey, 1953). The nutrient medium was the same as for fibroblasts except that the human ascites fluid was replaced by umbilical-cord serum.

HLM tissue is a transformed strain of human-liver cells from a 20-week male foetus (Sinclair & Leslie, 1959). It was grown in the same medium as HeLa tissue, but not as a monolayer.

Three days after the preparation of the fibroblast and HeLa-cell monolayers, either generally labelled L-[^{14}C]-tyrosine (usually 0·5 μc/tube) or DL-[1-^{14}C]leucine (usually 0·85 μc/tube) was added along with 1 ml. of fresh nutrient medium, and incubation was continued for a further 48 hr. The HLM-tissue cultures were incubated for 48 hr. with DL-[1-^{14}C]leucine (usually 1·7 μc/tube) in 1 ml. of fresh nutrient medium.

Separation of soluble proteins. After the end of incubation, the supernatant was decanted and the residual tissue was twice frozen in solid CO_2–ethanol and allowed to thaw. In this way the cell walls were ruptured. A small amount of quartz sand, 20 mg. of inactive carrier tyrosine or leucine and 2 ml. of 0·85% NaCl soln. were added, and the tissue was homogenized at room temperature within the roller tube by grinding for 5 min. with a rotating pestle. The cell debris and the sand were removed by centrifuging for 10 min. at 8000 rev./min., and the supernatant, together with the decanted nutrient medium, was subjected to ultrafiltration through a LSG 60 cellulose filter (Membranfilter-Gesellschaft, Göttingen) in a metal funnel under a pressure of N_2 of about 15 atmospheres (Manner, Broda & Kellner, 1957). The funnel was similar to the model MD 35–10 (Membranfilter-Gesellschaft). During the ultrafiltration, the metal was kept cool with ice cubes. The speed of filtration was about 2 ml./hr. The protein gel on top of the ultrafilter was washed five times with 5 ml. of 0·85% NaCl soln.

Experiments carried out with nutrient medium, in the absence of tissue, before and after incubation with the radioactive amino acid, showed that this procedure removed practically all radioactive compounds of low molecular weight from the protein (Table 1). High-molecular-weight compounds, such as soluble ribonucleic acid, will

Table 1. *Removal of low-molecular-weight radioactive materials from protein by ultrafiltration and washing*

Results in the absence of tissue; 0·5 μc of tyrosine was added.

	Not incubated		Incubated for 48 hr.	
Sample	Activity (disintegrations/min.)	Fraction of added tyrosine (%)	Activity (disintegrations/min.)	Fraction of added tyrosine (%)
Filtrate	Not estimated	Not estimated	Not estimated	Not estimated
First wash (water)	120 000	12	100 000	10
Second wash (water)	3 900	0·39	4 000	0·4
Third wash (water)	570	0·057	760	0·076
Fourth wash (water)	190	0·019	220	0·022
Fifth wash (water)	100	0·010	95	0·0095
Protein remaining	10	0·001	17	0·0017

Table 2. *Conditions of fractionation of serum proteins*

(*a*) Human serum and plasma; (*b*) chick serum and plasma.

Fraction	Concn. of ethanol (%, v/v) (*a*)	Concn. of ethanol (%, v/v) (*b*)	pH (*a*)	pH (*b*)	Ionic strength	Temperature
I (Fibrinogen)	10	8	7·2	7·2	0·13	−2°
II (β- and γ-Globulin)	22	20	7·2	7·2	0·08	−5
III (α-Globulin)	40	36	5·8	5·8	0·09	−5
IV (Albumin)	40	38	4·3	4·8	0·11	−5

stay with the protein. As far as they may contain radiocarbon in the given conditions, discrimination between them and the protein is left to the subsequent stages.

Fractionation with ethanol. After the removal of the low-molecular-weight compounds, the proteins were redissolved in 0·85% NaCl soln., and submitted to fractionation by selective precipitation with ethanol–water mixtures (Cohn *et al.* 1946). This method was favoured as the ethanol and the low temperature prevent the growth of bacteria. In extensive tests to adapt the procedure to preparation on a small scale, it was found that optimum conditions of fractionation were obtained by adjusting concentration of ethanol, pH, ionic strength and temperature as shown in Table 2. In practice, conditions (*b*) of Table 2 were used in the experiments with chick tissue though the nutrient medium contained mostly proteins of human origin.

pH was measured with a Beckman pH meter or universal indicator paper (E. Merck). The cooling mixture, ethanol–solid CO_2, was kept in a large Dewar flask. The protein solution was contained in a large metal centrifuge tube, immersed in the flask, and closed by a rubber bung carrying a short tube for attachment to a filter pump, and a small funnel with a sintered-glass disk (G1), which was kept under the surface of the solution. Suction was applied to introduce the ethanol–water mixtures and the buffer solutions slowly into the centrifuge tube through the porous disk. The solution in the centrifuge tube was mixed by sucking air through the disk. After each step precipitate and supernatant were separated by centrifuging at −2° or −5°.

Starch-gel electrophoresis. Electrophoresis in starch gel (Smithies, 1955, 1959) was used in every case as a test for the purity of the fractions obtained by ethanolic fractionation. The electrophoresis was carried out on a portion for 5–6 hr. at a potential gradient of about 7–14 v/cm., corresponding to roughly 200–400 v in total, with a current of 20–25 mA. The temperature was about 8°. The regions of the proteins were located by running non-radioactive serum in parallel with the active sample, and staining with Amido-black in water–methanol–acetic acid (50:50:10, by vol.).

Electrophoresis was also used to separate the β- and γ-globulin, which are obtained together in fractionation with ethanol. For the measurement of the activities of the individual proteins, they were eluted with 0·85% NaCl soln. after destroying the starch gel by freezing, and the starch was removed by centrifuging.

Radioimmunological experiments. Serum albumin obtained by fractionation with ethanol and checked for purity by electrophoresis (plus 5–10 mg. of inactive chick-serum albumin when anti-CSA was used) was mixed with the quantity of calibrated antiserum required to give optimum precipitation. The mixture was left standing for 2 hr. at room temperature and then kept in the refrigerator for 1–2 days; precipitate and supernatant were separated by centrifuging and the precipitate was washed successively with 2 ml. of 0·85% NaCl soln., 2 ml. of 5% trichloroacetic acid and 2 ml. of 50% ethanol. The supernatant was evaporated to dryness. Finally, the precipitate and the residue from the supernatant were subjected to combustion and the radioactivities were measured.

Reaction with ninhydrin. The proteins were hydrolysed by heating with 6 N-HCl for 48 hr. at 120°, and the acid was removed by evaporation under vacuum. The sample (i.e. hydrolysed or unhydrolysed protein) was kept at 100° for 30 min., together with 50 mg. of inactive leucine (carrier), 300 mg. of ninhydrin and 20 ml. of citrate buffer (pH 2·5). During heating, a current of CO_2-free air was passed through the mixture, and the CO_2 evolved was absorbed in NaOH after removal of water vapour and volatile aldehydes with conc. H_2SO_4 and solid CO_2–ethanol traps. $BaCO_3$ was precipitated from the NaOH solution and its radioactivity measured. The yield in this system was found, by volumetric estimation of CO_2 evolved from non-radioactive leucine, to be nearly quantitative.

Measurement of radioactivity. For the measurement of radioactivity, the proteins were subjected to combustion to CO_2 by a wet method (Van Slyke & Folch, 1940). A known amount of inactive carrier (15 mg. of phenanthrene) was added to the sample before combustion to obtain a sufficient amount of CO_2. This was absorbed in NaOH and precipitated with Ba^{2+} ions. Finally, the CO_2 was liberated from the $BaCO_3$ and introduced into the gas Geiger counter (Broda & Rohringer, 1954), where the ^{14}C β-particles are detected with 85% efficiency. The background of the counter, at various periods, was 40–50 min.$^{-1}$ The counting rates observed were converted into activities (disintegrations/min.) by the formula previously used (Suschny, Kellner, Broda, Figdor & Rücker, 1958).

The measurements of the radioactivities were often done with fairly small portions, often one-fifth or so, of the protein fractions, and the measured values had to be adjusted to the totals by multiplication. The reason was, in the experiments of Table 3, that only a small amount of protein could be introduced into the starch gel and, in those of Table 4, that a large part of each protein preparation was required to find the conditions of optimum precipitation. Therefore the relative statistical errors were high in samples of low activities, i.e. in the tails, though not in the main samples. For instance in column 2 of Table 3 (albumin) the measured activities (background deducted), with their standard deviations, were 155 ± 5, $10 \pm 2{\cdot}5$, $16 \pm 2{\cdot}5$, 2 ± 2, $5 \pm 2{\cdot}5$, $5 \pm 2{\cdot}5$ and $4 \pm 2{\cdot}5$ min.$^{-1}$ respectively. In Table 4 (B, *a*; HeLa cells with tyrosine), the figures were 19 ± 3 for

precipitate with anti-CSA, 190±5·5 for supernatant with anti-CSA, 206±5·5 for precipitate with anti-HSA and 14±3 for supernatant with anti-HSA. Zero activity has been entered in the tables when the activity actually measured was five disintegrations/min. above background or less, i.e. when the activity calculated to the whole of the original protein solution was less than about 75–150 disintegrations/min., depending on the portion measured.

RESULTS

Synthesis of serum proteins by tissue cultures. The activities of serum albumin and of α-, β- and γ-globulin were determined individually after incubation of chick-mesenchyma or HeLa tissue with radioactive L-tyrosine or DL-leucine. For this purpose, the fractions of serum albumin, of α-globulin and of (β+γ)-globulin were isolated by ethanolic precipitation, the fractions subjected separately to electrophoresis and the radiocarbon in the regions corresponding to the different serum proteins was measured in every electrophoresis gel. The intermediate regions between the bands of the proteins were also measured. It is clear from Table 3 that ethanolic fractionation had worked well, and that the radiocarbon was contained, both in the chick-mesenchyma and HeLa tissue, in all four fractions (albumin, α-, β- and γ-globulin).

In parallel control experiments, nutrient medium for mesenchyma, without tissue, was incubated with radioactive tyrosine, and medium for HeLa and HLM tissue with leucine. Solutions of the total serum proteins were obtained as with the tissues. The total activities of these proteins were less than 0·3% of those of the proteins produced in the presence of tissue and cannot be considered as significant.

Immunochemical identification of serum albumin. Immunochemical experiments were carried out with the serum-albumin fractions isolated by ethanolic precipitation and checked for purity by electrophoresis and staining. Measured portions of these fractions, obtained from all three kinds of tissue cultures incubated with radioactive amino acids, were treated with anti-CSA or with anti-HSA, and the radiocarbon in the precipitates and in the supernatants was determined in each (Table 4).

Table 3. *Activities in the electrophoresis gels of the protein fractions from ethanolic precipitation*

Region measured	Fractions: Albumin	α-Globulin	(β+γ)-Globulin
	(disintegrations/min.)		
(A) Chick mesenchyma was incubated for 48 hr. with 0·5 μc of L-tyrosine			
Albumin	5 120	640	440
Intermediate	330	420	0
α-Globulin	530	3 300	0
Intermediate	0	330	0
β-Globulin	0	0	3 840
Intermediate	0	0	520
γ-Globulin	0	0	3 150
(B) HeLa cells were incubated for 48 hr. with 0·85 μc of DL-leucine			
Albumin	15 300	940	1 330
Intermediate	2 000	620	920
α-Globulin	1 200	13 800	0
Intermediate	520	520	0
β-Globulin	0	490	13 580
Intermediate	0	0	1 480
γ-Globulin	0	0	12 200

Table 4. *Immunochemical identification of serum albumin*

Fraction tested	Treated with	Activity in: Precipitate	Supernatant
		(disintegrations/min.)	
(A) Chick mesenchyma			
(*a*) 0·5 μc of L-tyrosine (two incubations)			
Serum albumin	Anti-CSA	4 210, 4 370	305, 190
	Anti-HSA	495, 635	4 630, 3 660
α-Globulin	Anti-CSA	330	2 840
	Anti-HSA	300	2 400
(*b*) 0·85 μc of DL-leucine			
Serum albumin	Anti-CSA	14 300	1 380
	Anti-HSA	1 185	13 120
(B) HeLa cells			
(*a*) 0·5 μc of L-tyrosine			
Serum albumin	Anti-CSA	315	3 120
	Anti-HSA	3 400	225
(*b*) 0·85 μc of DL-leucine (three incubations)			
Serum albumin	Anti-CSA	1 800, 1 490, 1 150	13 000, 14 400, 12 300
	Anti-HSA	14 100, 12 400, 11 100	1 190, 1 590, 800
(C) HLM cells (1·7 μc of DL-leucine)			
Serum albumin	Anti-HSA	5 080	250

In blank experiments, run in parallel with those with tissue, the medium alone, in the absence of tissue, was incubated with radioactive amino acid under identical conditions, the soluble protein mixture was obtained by ultrafiltration and the albumin was precipitated from this mixture with anti-HSA. The activities of the precipitates were determined as usual, but were less than 2% of those of the albumin from comparable tissue experiments with chick-mesenchyma, HeLa or HLM tissue; they were considered as insignificant.

Table 4 shows that the radioactive protein of the serum-albumin fraction obtained with chick-mesenchyma tissue is precipitated specifically by anti-CSA but not by anti-HSA, whereas with the serum albumin from HeLa cells the opposite is true. The human-serum albumin from HLM-liver cells is also unambiguously identified as such.

Table 4 also includes some data obtained on treatment of α-globulin, isolated from chick mesenchyma by ethanolic precipitation and checked for purity by electrophoresis and staining, with anti-CSA and with anti-HSA. These data serve as additional controls both for the specificity of the antisera and for the sharpness of the separation between serum albumin and the globulins.

Action of ninhydrin on the radioactive protein. It has been pointed out above that the protein of the medium does not take up radioactivity. It remained to prove that the ^{14}C incorporated into the protein in the presence of tissue is present within the protein chain, that is, that the amino acid is bound within the protein in peptide linkage. This question was examined by the ninhydrin test (Winnick, 1950). Ninhydrin removes CO_2 from the carboxyl groups adjacent to free amino groups. Therefore radioactive CO_2 should be evolved from protein containing carboxyl-labelled amino acid on treatment with ninhydrin only after hydrolysis.

The ninhydrin test was applied (1) to a portion of the serum albumin from chick mesenchyma, isolated by ethanolic fractionation and tested for purity by electrophoresis and staining, and (2) to a portion of the total soluble protein from HeLa cells. Carboxyl-labelled DL-leucine (0·85 μc) was added to the medium before incubation in each case.

With HeLa protein only 4·9% of the radiocarbon of the protein (13 800 disintegrations/min.) was found in the CO_2 set free with ninhydrin without hydrolysis, but 90·3% of the radiocarbon in the protein (12 400 disintegrations/min.) after hydrolysis. Similarly, with chick mesenchyma only 3·7% of the radiocarbon of the serum albumin (2420 disintegrations/min.) was found in the CO_2 without hydrolysis, but 84·4% of the radiocarbon (3290 disintegrations/min.) after hydrolysis. Evidently the amino acid was held within the protein chain in α-peptide linkage, and was released and rendered capable of reacting with ninhydrin by hydrolysis.

Inhibition of protein synthesis by p-*fluorophenylalanine.* It was expected that new synthesis of proteins would be inhibited by *p*-fluorophenylalanine, an antagonist to the essential amino acid phenylalanine. If incorporation of leucine was unaffected, the results would point to a mechanism of incorporation of leucine other than protein synthesis afresh.

Monolayer cultures of HeLa tissue were used. The input was 600 000 cells/tube. Incubation with 0·85 μc of DL-leucine lasted 48 hr. Some of the tubes were used for the determination of the radioactivity of the albumin after fractionation with ethanol and examination by electrophoresis. In eight to ten parallel tubes, the tissue was redispersed (converted into a cell suspension) with trypsin after 48 hr., and the cells were counted again.

In fact, heavy inhibition of incorporation into albumin was observed. However, the number of cells per tube at the end of incubation was also much reduced compared with the controls (Table 5). Fluorophenylalanine acts as a potent cell poison.

The numbers of cells during the 48 hr. of the experiment and the effects of the antagonist on their biosynthetic capacities are not known. Although in the circumstances no definite conclusion about the mechanism can be drawn, the parallel between the incorporation of leucine into serum albumin and the number of surviving cells suggests that the decrease in leucine incorporation is due to the drop in the formation of new serum albumin. Certainly there is no evidence that leucine is incorporated into preformed albumin. Such evidence would, of course, run counter to our general ideas about protein biosynthesis.

DISCUSSION

The method here described gives unambiguous information about the autonomous production of individual proteins by particular kinds of tissue. The new data show that chick-embryo-mesenchyma tissue produces chick-serum albumin, and both human-tumour HeLa tissue and transformed

Table 5. *Action of* p-*fluorophenylalanine on HeLa cells*

Concn. of fluorophenylalanine (mM)	Radioactivity of albumin (disintegrations/min.)	Mean cell number after 48 hr. (% of control)
0	6480	100
0·01	5720	75·5
0·1	2960	33·8
1·0	650	9·2

human-liver HLM tissue produce human-serum albumin. Our conclusion that some extrahepatic tissues make serum albumin contradicts a widely held opinion. For instance it has been stated (Peters, 1957) that 'failure to find labelled carbon in the albumin from the particulate fractions of kidney, spleen, intestinal mucosa and heart after injection (of labelled amino acid) *in vivo* supports the wealth of previous evidence that the liver is the sole source of serum albumin (Miller, Bly, Watson & Bale, 1951; Popper & Schaffner, 1957; Dancis, Braverman & Lind, 1957)'. See also Miller, Bly & Bale, 1954.

We have not determined to what extent the amount of the serum proteins increases during incubation, i.e. whether there is a net production of these proteins. Eagle, Piez, Fleischman & Oyama (1959) have shown that strong incorporation of labelled amino acids into proteins can take place in tissue culture without net production of proteins. This 'dynamic' renewal of proteins can proceed at the rate of 1%/hr., whereas in conditions of maximum growth the amount of protein increases by 4%/hr. In every case, the incorporation takes place intracellularly. The mechanism of renewal is presumably identical with the mechanism for new synthesis.

Though it has now been shown that the machinery for making serum albumin exists within chick-embryo-mesenchyma and HeLa-tumour cells, it is not clear to what extent it is used *in vivo*. The serum-albumin production by the tissue *in vitro* can be estimated only crudely from the experiments here reported. We take Table 4 (B, *b*; HeLa cells with leucine) as a basis, and assume that the recovery of serum albumin after incubation with leucine was of the order of 50%, i.e. that the total activity of the serum albumin was about 25 000 disintegrations/min. Then it is found that about 1·32% of the initial amount of radioactive leucine ($1{\cdot}9 \times 10^6$ disintegrations/min.), i.e. 2·64% of the L-leucine, had been utilized to build up serum albumin.

For a calculation of the weight of this newly formed albumin, the effective specific activity of the free leucine is required. An approximate estimate is possible on the basis that the medium (which contains about one-half of buffered salt solution) has half the free leucine concentration of serum. This is about 25 μg./ml. (Flaschenträger & Lehnartz, 1954). The concentration of the leucine will not change much during incubation, as essential amino acids are not formed anew, and, on the other hand, only a small proportion of the leucine was used. If further the crude assumption is made that the specific activity of the newly incorporated leucine is about half that of the free leucine in the medium (Loftfield & Harris, 1956; Steiner & Anker, 1956; Askonas & Humphrey, 1958), the mass of the leucine incorporated into serum albumin works out as

$$12{\cdot}5 \times 0{\cdot}0264 \times 2 = 0{\cdot}66\ \mu\text{g}.$$

With a leucine content (by weight) of human-serum albumin of 11%, deduced from data given by Hughes (1954) and Peters, Logan & Sanford (1958), the quantity of albumin newly formed in 48 hr. is computed as 6 μg./roller tube.

This amount of new albumin is to be compared with the mass of the tissue. The mean dry weight of one HeLa cell is taken as $7{\cdot}15 \times 10^{-4}$ μg. (Leslie, Fulton & Sinclair, 1957; see, however, Salzman, 1959), and each roller tube contained initially about 6×10^5 cells, i.e. the dry weight of tissue was 430 μg./tube. If the change in the number of cells during incubation, which was not large, is neglected, the order of magnitude of the production of serum albumin in 48 hr. is obtained as 14 mg./g. of dry HeLa tissue, and in 1 hr. it was about 290 μg./g.

The protein content of one HeLa cell is given as $4{\cdot}8 \times 10^{-4}$ μg. (Leslie *et al.* 1957), and 6×10^5 cells therefore contain 290 μg. of protein. Thus the serum albumin produced in 48 hr., whether by net production or by renewal, corresponds to 2%, compared with the total protein content of the HeLa tissue. All the serum proteins taken together incorporate about 3·5 times as much leucine as the albumin alone (Table 3).

If similar calculations are carried out for chick-mesenchyma tissue (Table 4, A, *b*), and the weight of the cell is assumed to be two-thirds that of a HeLa cell (Manner *et al.* 1957), an hourly rate of albumin production of about 500 μg./g. of dry tissue is reached. The order of magnitude agrees with that given for the production of serum albumin by sections of chicken liver, i.e. 120 μg./g. of wet liver/hr. (Peters & Anfinsen, 1950). The production rate per unit weight is, however, larger than that calculated from the data by Sterling (1951) for whole man, or from the data by Jeffay, Winzler & Donnelly (1958) for rats.

Serum globulins are known to be formed, to varying extents, by several kinds of tissue. This applies also to the γ-globulins, and more particularly to the antibodies (Askonas & White, 1956; Askonas, Humphrey & Porter, 1956; Stavitsky, 1958; G. J. Thorbecke, personal communication). In the present investigation it has been shown that α-, β- and γ-globulin are formed by chick-mesenchyma tissue, by HeLa-tumour tissue and by HLM transformed-liver tissue. No qualitative differences between mesenchyma and HaLa tissue have been observed so far by us in respect to the production of any of the serum proteins.

Immunological work with the serum globulins is under way. In this work, the absolute rates of

production of different proteins are measured with better precision. The precision is improved by (1) adjusting the specific activity of the amino acid to a known value by adding known excess amounts of the non-radioactive amino acid before incubation, and by (2) determining the yield in the isolation of the protein fractions by isotope dilution after the addition of ^{131}I-labelled proteins.

SUMMARY

1. A method is described to establish the autonomous production of individual serum proteins by tissue in culture. The tissue is incubated with nutrient medium containing a ^{14}C-labelled amino acid (tyrosine or leucine), the soluble proteins are separated and purified by ultrafiltration, fractionation with ethanol and starch-gel electrophoresis, and the radioactivity of the individual proteins is measured.

2. The proteins of the nutrient medium contained no radiocarbon after incubation with the radioactive amino acid in the absence of tissue.

3. When tissue was incubated with medium and carboxyl-labelled leucine, and the protein after isolation was treated with ninhydrin, appreciable quantities of radioactive carbon dioxide were evolved only if the protein had been hydrolysed.

4. By these methods, the incorporation of radioactive tyrosine or leucine into serum albumin as well as into α-, β- and γ-globulin was demonstrated with chick-embryo-mesenchyma tissue, human-tumour HeLa tissue and human transformed-liver HLM tissue.

5. A crude estimate of the rates of production gave the result that 15 mg. of albumin was produced/g. of dry HeLa tissue in 48 hr., and 24 mg./g. in chick-mesenchyma tissue.

6. The identity of the labelled serum albumin was confirmed immunologically by treatment with antisera to chick- and human-serum albumin. In the experiments with chick mesenchyma, the bulk of the radiocarbon was found in the precipitate only after reaction with anti-chick serum albumin, whereas with HeLa and HLM tissue the opposite was true.

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REFERENCES

Abdel-Samie, Y. M., Broda, E., Kellner, G. & Zischka, W. (1959). *Nature, Lond.*, **184**, 361.

Abdel-Tawab, G. A., Broda, E. & Kellner, G. (1959). *J. Chromat.* **2**, 99.

Askonas, B. A. & Humphrey, J. H. (1958). *Biochem. J.* **70**, 212.

Askonas, B. A., Humphrey, J. H. & Porter, R. R. (1956). *Biochem. J.* **63**, 412.

Askonas, B. A. & White, R. G. (1956). *Brit. J. exp. Path.* **37**, 61.

Boyd, W. C. (1945). *J. Amer. chem. Soc.* **67**, 1035.

Broda, E. & Rohringer, G. (1954). *Z. Elektrochem.* **58**, 634.

Cohn, E. J., Strong, L. E., Hughes, W. L. jun., Mulford, D. J., Ashworth, J. N., Melin, M. & Taylor, H. L. (1946). *J. Amer. chem. Soc.* **68**, 459.

Dancis, J., Braverman, N. & Lind, J. (1957). *J. clin. Invest.* **36**, 398.

Eagle, H., Piez, K. A., Fleischman, R. & Oyama, V. I. (1959). *J. biol. Chem.* **234**, 592.

Flaschenträger, B. & Lehnartz, E. (1954). *Physiologische Chemie, Erster Teil, Bandteil a*, p. 344. Berlin: Springer-Verlag.

Gey, G. O. (1933). *Amer. J. Cancer*, **17**, 752.

Gey, G. O., Coffman, W. D. & Kubicek, M. T. (1952). *Cancer Res.* **12**, 264.

Heidelberger, M. & Kendall, F. E. (1935). *J. exp. Med.* **62**, 697.

Heidelberger, M. & Kendall, F. E. (1937). *J. exp. Med.* **65**, 647.

Hughes, W. L. (1954). *The Proteins*, vol. 2, part B, p. 687. New York: Academic Press Inc.

Jeffay, H., Winzler, R. J. & Donnelly, J. S. (1958). *J. biol. Chem.* **231**, 101.

Kellner, G. & Stockinger, L. (1957). *Arch. int. Pharmacodyn.* **110**, 259.

Leslie, I., Fulton, W. C. & Sinclair, R. (1957). *Biochim. biophys. Acta*, **24**, 365.

Loftfield, R. B. & Harris, A. (1956). *J. biol. Chem.* **219**, 151.

Manner, G., Broda, E. & Kellner, G. (1957). *Mh. Chem.* **88**, 896.

Miller, L. L., Bly, C. G. & Bale, W. F. (1954). *J. exp. Med.* **99**, 133.

Miller, L. L., Bly, C. G., Watson, M. L. & Bale, W. F. (1951). *J. exp. Med.* **94**, 431.

Pappenheimer, A. M. (1940). *J. exp. Med.* **71**, 263.

Peters, T. (1957). *J. biol. Chem.* **229**, 659.

Peters, T. & Anfinsen, C. B. (1950). *J. biol. Chem.* **182**, 171.

Peters, T., Logan, A. C. & Sanford, C. A. (1958). *Biochim. biophys. Acta*, **30**, 88.

Popper, H. & Schaffner, F. (1957). *Liver: Structure and Function*. New York: McGraw-Hill Book Co. Inc.

Salzman, N. P. (1959). *Biochim. biophys. Acta*, **31**, 158.

Scherer, W. F., Syverton, J. T. & Gey, G. O. (1953). *J. exp. Med.* **97**, 695.

Sinclair, R. & Leslie, I. (1959). *Biochim. biophys. Acta*, **32**, 58.

Smithies, O. (1955). *Biochem. J.* **61**, 629.

Smithies, O. (1959). *Biochem. J.* **71**, 585.

Stavitsky, A. B. (1958). *Brit. J. exp. Path*, **39**, 661.

Steiner, D. F. & Anker, H. S. (1956). *Proc. nat. Acad. Sci., Wash.*, **49**, 580.

Sterling, K. (1951). *J. clin. Invest.* **30**, 1238.

Suschny, O., Kellner, G., Broda, E., Figdor, B. & Rücker, W. (1958). *Expt. Cell Res.* **14**, 316.

Van Slyke, D. D. & Folch, J. (1940). *J. biol. Chem.* **136**, 509.

Winnick, T. H. (1950). *Arch. Biochem.* **27**, 65.

Biochem. J. (1960) **75**, 216

Further Degradation Products of Cephalosporin C

ISOLATION AND SYNTHESIS OF 2-(4-AMINO-4-CARBOXYBUTYL)THIAZOLE-4-CARBOXYLIC ACID

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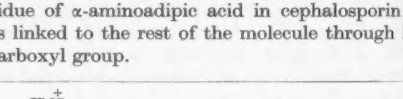
Abraham & Newton (1956) showed that cephalosporin C closely resembled (D-4-amino-4-carboxybutyl)penicillin (cephalosporin N) in some of its chemical properties but that it differed from a penicillin in others. On hydrogenolysis with Raney nickel and subsequent hydrolysis with acid cephalosporin C yielded DL-valine and D-α-aminoadipic acid, together with some L-alanine and glycine. On acid hydrolysis alone it yielded D-α-aminoadipic acid but no penicillamine (β-mercaptovaline). It thus appeared that cephalosporin C contained the carbon–nitrogen skeleton of penicillamine, but that it did not contain the characteristic 4-carboxy-5:5-dimethylthiazolidine ring of the true penicillins.

This paper describes two degradation products of cephalosporin C which are derived from the side chain and part of the nucleus to which the latter is attached. One product is apparently δ-amino-δ-carboxyvalerylglycine (I), which has been obtained earlier from cephalosporin N (Newton & Abraham, 1954; Abraham & Newton, 1954). The other is 2-(D-4-amino-4-carboxybutyl)thiazole-4-carboxylic acid (II) and results from a type of fission that has not been observed with cephalosporin N or other penicillins.

When cephalosporin C was heated in 0·1N-hydrochloric acid at 100° for 16 min., carbon dioxide was evolved and a complex mixture of ninhydrin-positive degradation products was formed. Analysis of the mixture by electrophoresis on paper, followed by chromatography in butan-1-ol–acetic acid–water (Woiwod, 1949), showed that at least three acidic substances, three neutral substances and two basic substances were present. One of the acidic substances corresponded in behaviour with α-aminoadipic acid. All the remaining substances were labile on treatment with acid under more vigorous conditions, being destroyed in N-hydrochloric acid at 100° in 30 min.

The neutral products formed on hydrolysis of cephalosporin C in 0·1N-hydrochloric acid for 16 min. were separated from acidic material by passage through a column of Dowex 1 (×10) (acetate form), and then from basic material by passage through a column of Amberlite XE-64 (pyridine form). Oxidation of the resulting neutral fraction with silver oxide yielded a small amount of an acidic substance which was purified by chromatography on Dowex 1 (×10) (acetate form). This substance appeared to be homogeneous, except for contamination with a trace of α-aminoadipic acid, when examined by electrophoresis and chromatography on paper in a number of different systems (see Experimental). In these systems it was indistinguishable from an authentic sample of D-δ-amino-δ-carboxyvalerylglycine synthesized by Abraham & Newton (1954). On acid hydrolysis it yielded approximately equal amounts of two substances which were indistinguishable from glycine and α-aminoadipic acid respectively. It was concluded that the substance from cephalosporin C was δ-amino-δ-carboxyvalerylglycine (I). Earlier work (Abraham & Newton, 1956) had indicated that the residue of α-aminoadipic acid in cephalosporin C was linked to the rest of the molecule through its δ-carboxyl group.



(I)

When a solution of cephalosporin C in aqueous pyridine (pH 5–7) was kept at 37° a new substance with antibacterial activity was formed. On paper electrophoresis at pH 7 this substance behaved as though it had no net charge. It was subsequently shown to be one of a family of compounds (named the cephalosporin C_A family) whose different members are formed by reaction of cephalosporin C with different heterocyclic bases (Hale, Abraham & Newton, 1958; Abraham & Newton, 1958). After the reaction in aqueous pyridine had been allowed to proceed for 72 hr., some cephalosporin C remained unchanged and migrated towards the anode when the mixture of products was subjected to electrophoresis on paper at pH 7 (Abraham & Newton, 1958). A second acidic substance, which showed no detectable activity against *Staphylococcus aureus* (N.C.T.C. 6571) or *Salmonella typhi* (strain 'Mrs S', Felix & Pitt, 1935), was revealed when the paper was sprayed with ninhydrin. This

substance migrated slightly faster than cephalosporin C, from which it was just resolved. It was also formed when the sodium salt of cephalosporin C was kept in water (pH 7) at 37°, although no cephalosporin C_A was produced under these conditions.

The new acidic substance was isolated from the products obtained when cephalosporin C was kept in aqueous pyridine by chromatography of the mixture on a column of Dowex 1 (×10) (acetate form). Cephalosporin C_A passed rapidly through the column. On elution with 0·5N-acetic acid the new substance emerged in a sharp band which preceded, and was well separated from, a band due to cephalosporin C.

The substance isolated in this way was readily soluble in water at pH 7·5, but separated in crystalline form when the solution was adjusted to pH 3·8. It showed $[\alpha]_D^{20} - 17{\cdot}6°$ in N-hydrochloric acid. Elementary analysis indicated that a possible molecular formula was $C_9H_{12}O_4N_2S$. The results of electrometric titration were consistent with the presence of a basic group (pK_a 9·9) and two acidic groups (pK_a about 2·6 and 4·0 respectively) in a compound with this molecular formula. The ultraviolet absorption of the substance showed a maximum at 237 mμ in water (log ε 3·78) and at 233 mμ in N-hydrochloric acid (log ε 3·88). The substance appeared to be unchanged by treatment with hot 6N-hydrochloric acid or with 0·1N-sodium hydroxide at room temperature.

These properties, when considered in conjunction with the information relating to the structure of cephalosporin C that had been obtained by Abraham & Newton (1956), suggested that the new substance was the thiazole (II). The absorption of the substance in ultraviolet light was similar to that recorded by Brookes, Fuller & Walker (1957) for 2-(1-amino-2-methylpropyl) thiazole-4-carboxylic acid hydrochloride obtained from micrococcin P. The change in $\lambda_{max.}$ from 237 mμ in water to 233 mμ in N-hydrochloric acid could be attributed to protonization of the heterocyclic nitrogen atom. Dr James Walker informed us that no such change in $\lambda_{max.}$ occurred with 2-(1-amino-2-methylpropyl)-thiazole-4-carboxylic acid, but suggested that in this case protonization of the heterocyclic nitrogen atom is hindered by the proximity of a positively charged amino group.

Support for structure (II) was obtained from an examination, by electrophoresis and chromatography on paper, of the products formed when the substance was subjected to hydrogenolysis under two different conditions, followed by hydrolysis with hot 6N-hydrochloric acid. Hydrogenolysis with hydrogen in the presence of an excess of Adams catalyst led to products which appeared to be glycine (III), alanine (IV), α-aminoadipic acid (V), lysine (VII) and norleucine (VIII) respectively. Two other ninhydrin-positive products were also formed. The first showed a low R_F (R_{Gly} 0·56) [the abbreviations in this subscript and in those below are for amino acids; see *Biochem. J.* (1957), **66**, 8] in butan-1-ol–acetic acid–water and no net charge at pH 7, properties which suggested that it might be the diaminodicarboxylic acid (VI). The behaviour of the second, which showed R_{Lys} 1·4 and migrated slightly less rapidly than lysine towards the cathode at pH 7, suggested that it might be ε-*N*-ethyl-lysine (IX). Hydrogenolysis with Raney nickel led to alanine, a neutral substance with R_{Gly} 0·56 and a third substance with R_{Val} 0·92. The third substance showed the same R_F as pipecolic acid (X). Like the latter, it gave a violet spot which faded to brown–grey when sprayed on paper with ninhydrin, and a green spot when sprayed with a solution of isatin in acetone (Grobbelaar, Zacharius & Steward, 1954). All these products could be accounted for if hydrogenolysis involved fission at two or more points of the thiazole ring, as indicated by the broken lines in (II). The type of fission responsible for the formation of pipecolic acid would be analogous to that which yields isoleucinol from the *N*-terminal 2-(1-amino-2-methylbutyl)thiazoline fragment of bacitracin A (Lockhart, Abraham & Newton, 1955).

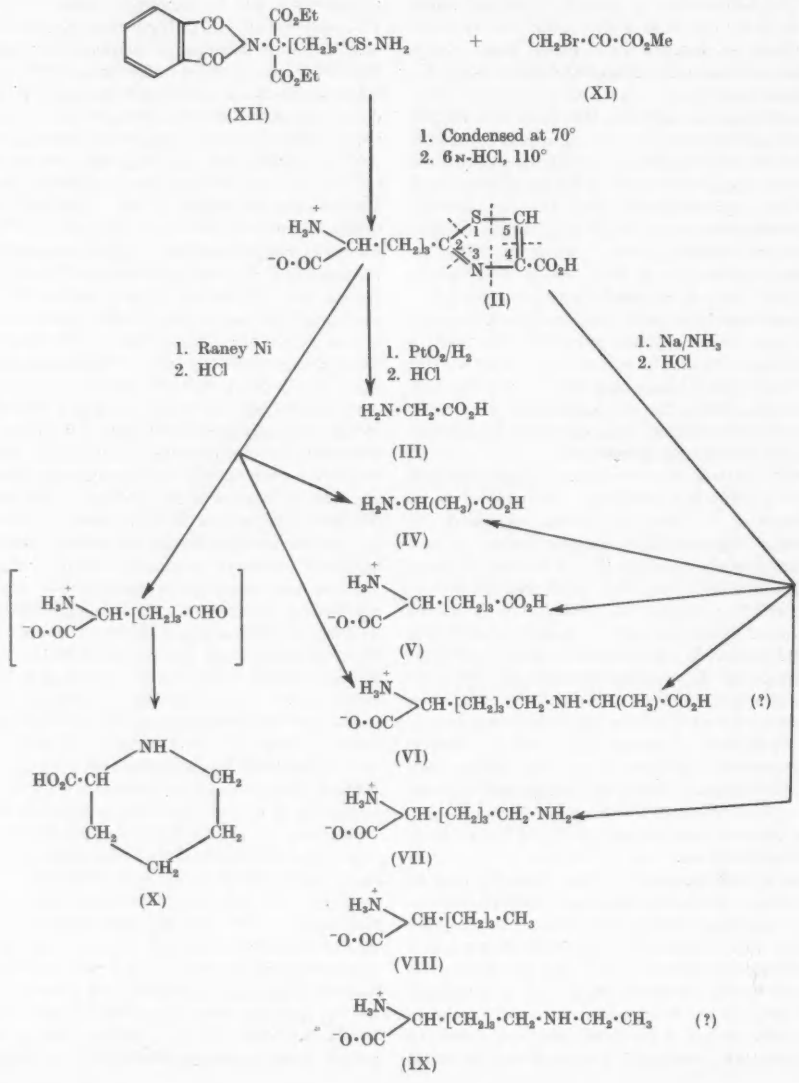
At this stage we were indebted to Dr James Walker for information about a method for the structural study of thiazoles which consisted of reduction with sodium in liquid ammonia followed by acid hydrolysis of the product (Brookes, Clark, Majhofer, Mijović & Walker, 1960). The application of this method to the substance from cephalosporin C yielded products which behaved like alanine, α-aminoadipic acid and lysine respectively, and also a neutral product with R_{Gly} 0·56 in butan-1-ol–acetic acid–water. Fission of (II) to yield these products was consistent with the results of Brookes *et al.* (1960), who obtained alanine and valine from 2-(1-amino-2-methylpropyl)thiazole-4-carboxylic acid and alanine and *N*-benzylalanine from 2-phenylthiazole-4-carboxylic acid. The fission of 2-phenylthiazole-4-carboxylic acid to yield *N*-benzylalanine would correspond with the fission of the thiazole (II) to yield the compound with R_{Gly} 0·56 if the latter had the structure (VI).

2-(DL-4-Amino-4-carboxybutyl)thiazole-4-carboxylic acid (II) was then synthesized by condensation of methyl bromopyruvate (XI) with the thioamide (XII), or with the corresponding acetamido compound, and hydrolysis of the product.

The thioamide (XII) was prepared from the corresponding phthalimidonitrile (Sörensen, 1903–06) by heating the latter with liquid hydrogen sulphide at 70° in an autoclave (cf. Erne & Erlenmeyer, 1948), and was obtained in crystalline form

after countercurrent distribution in the system benzene–methanol–water (1:1:1, by vol.). Its infrared-absorption spectrum in chloroform showed bands at 1785 and 1722 cm.$^{-1}$ which could be attributed to the phthalimido group (cf. Sheehan, Henery-Logan & Johnson, 1953). Its ultraviolet-absorption spectrum in ethanol showed one maximum at 220 mμ (log ϵ 4·62) attributable to the phthalimido group, and one at 269 mμ (log ϵ 4·02) attributable to the thioamide. Burawoy (1939) reported $\lambda_{max.}$ 265 mμ, log ϵ 4·11 for thioacetamide.

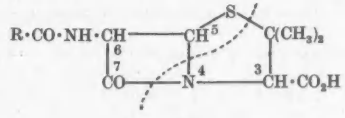
The corresponding acetamidonitrile was prepared from diethyl acetamidomalonate and γ-chlorobutyronitrile and converted into the thioamide in a similar manner. Condensation of either thioamide with methyl bromopyruvate in ethanol at 70° yielded a product from which the optically



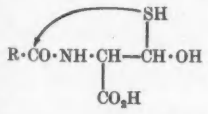
inactive thiazole (II) was formed on hydrolysis with acid.

The synthetic thiazole was indistinguishable from the substance from cephalosporin C when the two products were compared by electrophoresis or chromatography on paper under a variety of conditions. The ultraviolet-absorption spectra of the two compounds (in water or in N-hydrochloric acid) were very similar. The substance from cephalosporin C was racemized by treatment with acetic anhydride under conditions similar to those used by du Vigneaud & Meyer (1932) for the racemization of amino acids. The optically inactive product (m.p. 233–237°) and the synthetic thiazole (m.p. 235–237°) showed no depression in melting point when mixed. X-ray powder photographs of the optically inactive product and the synthetic thiazole were kindly taken by Mrs S. Cole and Dr Dorothy Hodgkin and reported to be identical.

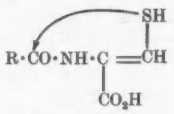
The stable thiazole (II) cannot be present in cephalosporin C itself, since the latter readily yields α-aminoadipic acid on treatment with hot dilute acid. The thiazole ring may therefore be formed by a rearrangement which accompanies a hydrolytic cleavage of cephalosporin C in neutral solution. However, the formation of (I) and (II) suggests that cephalosporin C contains a sequence of atoms which is identical with, or very similar to, that in the *N*-acylcysteine fragment of the true penicillins (XIII). It thus strengthens earlier evidence that cephalosporin C is biogenetically related to cephalosporin N. The sulphur atom and C-5, C-6 and C-7 in the fragment of (XIII) to the left of the dissection shown by the broken line could correspond with a fragment of cephalosporin C which yields the sulphur atom, C-5, C-4 and the carboxyl group respectively of (II). The formation of (I) after acid hydrolysis and oxidation with silver oxide is consistent with the assumption that carbon atoms in cephalosporin C which correspond to C-7 and C-5 in (XIII) form part of a latent labile carboxyl group and a potential aldehyde group respectively. Fission of a sulphur–carbon bond in cephalosporin C and other hydrolytic changes might then lead to transient intermediates such as (XIV) or (XV) from which the thiazole (II) could be formed by intramolecular condensation.



(XIII)



(XIV)



(XV)

EXPERIMENTAL

Elementary analyses were by Weiler and Strauss. Infrared spectra were measured with a Perkin–Elmer Double Beam Photometer Model 21.

Electrophoresis and chromatography on paper. Electrophoresis on Whatman no. 1 paper (14 v/cm. for 2·5 hr.) was carried out as described by Newton & Abraham (1954) in collidine acetate buffer (0·05 M to acetate), pH 7, and in 10% (v/v) acetic acid, pH 2·3. Paper chromatograms were run on Whatman no. 1 paper in the following solvent systems: A, butan-1-ol–acetic acid–water (4:1:4, by vol.) (Woiwod, 1949); B, 80% (w/w) phenol in an atmosphere saturated with 50% (v/v) acetic acid (Dent, 1948); C, butan-1-ol saturated with aq. 0·1 N-HCl; D, butan-2-ol saturated with 3% aqueous NH_3; E, propan-1-ol–water (7:3, v/v); F, triethylamine–acetone–water (1:16:3, by vol.) (Wright & Stadtman, 1956).

In some experiments electrophoresis at pH 7 was followed by chromatography in system A, the solvent being run in a direction perpendicular to that of the current.

Electrometric titrations. These were carried out in the manner described by Newton & Abraham (1954).

Isolation of δ-amino-δ-carboxyvalerylglycine from cephalosporin C. A solution of cephalosporin C (sodium salt) (100 mg.) in 0·1 N-HCl (5 ml.) was heated at 100° for 16 min. It was then evaporated to dryness in a rotary evaporator (Craig, Gregory & Hausmann, 1950) and the residue kept in a vacuum desiccator overnight in the presence of solid KOH. The dry residue was dissolved in water (2 ml.) and the solution added to a column of Dowex 1 (×10) (5 cm. × 1 cm., 200–400 mesh) in the acetate form. On elution with water, neutral and basic ninhydrin-positive material passed rapidly through the column and emerged in 6 ml. of percolate. The latter was evaporated to dryness *in vacuo* and the residue dissolved in water (2 ml.) and added to a column of Amberlite XE-64 previously washed with an excess of aq. N-pyridine and then with water (50 ml.). On elution with water, neutral ninhydrin-positive material emerged from the column in the first 14 ml. of percolate. The latter yielded 30 mg. of residue on evaporation to dryness *in vacuo*.

The neutral residue was dissolved in water (5 ml.) and the solution stirred with Ag_2O (freshly prepared from 340 mg. of $AgNO_3$) at 80° for 1·5 hr. Ag^+ ions were removed from the supernatant by addition of N-HCl (to pH 4) and the clear solution obtained after centrifuging was evaporated to dryness *in vacuo*. The residue (15 mg.) was dissolved in water (1 ml.) and added to a column of Dowex 1 (×10) (5 cm. × 1 cm., 200–400 mesh) in the acetate form. Neutral material was eluted with water (2 ml.). Elution was then continued with 0·5 N-acetic acid, 1 ml. fractions being collected. An acidic ninhydrin-positive substance (3 mg.) emerged in fractions 5 and 6.

This substance was indistinguishable from synthetic D-δ-amino-δ-carboxyvalerylglycine (Abraham & Newton, 1954)

when subjected to electrophoresis or chromatography on paper under various conditions. On electrophoresis in collidine acetate (pH 7) both products migrated somewhat less far (6·8 cm.) towards the anode than α-aminoadipic acid (8·0 cm.) and on electrophoresis in 10% (v/v) acetic acid they migrated slightly less far (4·2 cm.) towards the cathode than α-aminoadipic acid (5·3 cm.). On chromatography in butan-1-ol–acetic acid–water and in phenol–acetic acid both products showed R_F values of 0·16 and 0·31 respectively. Hydrolysis of the product from cephalosporin C with N-HCl at 110° for 16 hr. yielded material which showed two ninhydrin-positive spots of similar intensity after electrophoresis and chromatography on paper. One of these spots was indistinguishable from a spot due to glycine, and the other from a spot due to α-aminoadipic acid, after electrophoresis at pH 7 and chromatography in systems A and F.

Isolation of 2-(D-4-*amino*-4-*carboxybutyl*)*thiazole*-4-*carboxylic acid from cephalosporin C.* To a stirred solution of cephalosporin C (sodium salt) (1·0 g.) in water (50 ml.) was added Dowex 50 (×8) (200–400 mesh, H form) until the pH fell to 2·5. About 680 mg. of damp-dry resin was required. The resin was removed by filtration and pyridine (4 ml.) was added to the resulting solution of cephalosporin C (free acid). The mixture was kept at 37° for 72 hr. and then freeze-dried. The residue was stirred with acetone (about 50 ml.) to remove traces of pyridine and the remaining solid was separated by centrifuging and freed from acetone *in vacuo*. The resulting powder (750 mg.) was dissolved in water (2 ml.) and added to a column of Dowex 1 (×10) (25 cm. × 2 cm., 200–400 mesh) in the acetate form. Neutral material was eluted with water, 5 ml. fractions being collected. The neutral cephalosporin C_A (Abraham & Newton, 1958; Hale *et al.* 1958) emerged in fractions 4–6. Elution was then continued with 0·5 N-acetic acid, 25 ml. fractions being collected. A sharp band of ninhydrin-positive material emerged in fractions 25–30. A broader band due to unchanged cephalosporin C emerged in fractions 60–80. After neutralization with NaOH this yielded 165 mg. of cephalosporin C (sodium salt).

Fractions 25–30 were combined and the solution was evaporated to dryness *in vacuo*. The remaining white microcrystalline solid (46 mg.) was mixed with water (0·2 ml.) and aq. N-NH_3 added until the solid had completely dissolved (pH 8). 4 N-Acetic acid was then added to the clear solution in an amount sufficient to lower its pH to about 3·8. Within a few seconds the product began to separate in crystalline form. After 2 hr. the mixture was filtered and the product washed with a little water and dried in a vacuum desiccator (38·6 mg.). The off-white product lost no further weight on drying at 56° in a high vacuum. It had m.p. 245–246° (decomp.), $[\alpha]_D^{20}$ −17·6° in N-HCl (*c*, 1·3) (Found: C, 43·8; H, 5·0; N, 10·9; S, 12·3%; equiv. 250. $C_9H_{12}O_4N_2S$ requires C, 44·2; H, 4·9; N, 11·5; S, 13·1%; equiv. 244). The presence of a small amount of pigmented impurity, which was not removed by recrystallization, may have been responsible for the low values found for N and S. The pigment was lost during procedures, described below, that were used to obtain the compound in a racemic form, and satisfactory analytical data were obtained with the racemate. The ultraviolet-absorption spectrum of the optically active product showed $\lambda_{max.}$ 237 mμ (log ε 3·78) in water and $\lambda_{max.}$ 233 mμ (log ε 3·88) in N-HCl.

The product was poorly soluble in water between pH 3 and 5, but was readily soluble in N-HCl or at pH values higher than 7. Electrometric titration in water at 20° indicated that it contained ionizable groups with pK_a values of about 2·6, about 4·0, and 9·9 respectively. When subjected to electrophoresis on paper at pH 7 it migrated less far (5·5 cm.) towards the anode than α-aminoadipic acid (8·0 cm.) but further than cephalosporin C (4·8 cm.) (spots revealed by ninhydrin). On paper chromatograms run in system A it showed an R_F slightly greater than that of α-aminoadipic acid, the two compounds just being resolved when run together. These properties were unchanged by keeping the product 16 hr. in 0·1 N-NaOH at 20° or in N-HCl at 105°.

Degradations of the thiazole from cephalosporin C. (*a*) *Hydrogenolysis in the presence of platinum dioxide.* A solution of the thiazole (5 mg.) in 0·5 N-acetic acid (15 ml.) was shaken with Adams catalyst (25 mg., L. Light and Co. Ltd.) under H_2 at room temp. for 22 hr. The mixture was filtered and the filtrate evaporated to dryness. The residue was heated in 6 N-HCl (0·5 ml.) for 16 hr. at 105° and the solution evaporated to dryness. The resulting material was then examined by electrophoresis on paper at pH 7, followed by chromatography on paper in system A. Spots were coloured with ninhydrin, and in the following account the relative intensity of the spots is indicated by the number of + signs in parentheses. Of the products which showed no net charge at pH 7 three had R_F values corresponding to glycine (+), alanine (+ + +) and norleucine (+) respectively and a fourth substance (+ +) which was possibly (VI), showed R_{Gly} 0·56. Two basic products were observed, one of which corresponded to lysine (+ + +) in mobility and R_F and another (+) which migrated somewhat less far (10·0 cm.) than lysine (12·0 cm.) towards the cathode and showed R_{Lys} 1·4. The latter was conceivably (IX). One acidic product was observed (+ + +) which corresponded in mobility and R_F with α-aminoadipic acid.

(*b*) *Hydrogenolysis with Raney nickel.* The thiazole (6 mg.) was mixed with water (1 ml.) in a tube fitted with a reflux condenser and 0·1 N-NaOH added until a clear solution was obtained (pH 7·5). About 50 mg. of Raney nickel (Pavlic & Adkins, 1946) was added and the tube immersed in an oil bath at 160° for 20 min. The insoluble material was spun down and washed twice with water. The combined supernatant and washings were shaken twice with 1 vol. of 1% (w/v) oxine in $CHCl_3$ and then twice with 1 vol. of $CHCl_3$ alone. The aqueous solution was evaporated to dryness *in vacuo* and the residue (5 mg.) heated in 6 N-HCl (0·5 ml.) at 110° for 16 hr. The HCl was removed *in vacuo* and the residue examined by electrophoresis on paper at pH 7, followed by chromatography on paper in system A. Three spots were revealed by ninhydrin, all of which were due to substances that showed no net charge at pH 7. The R_F of one spot (+ + +) corresponded with that of alanine. A second spot (+ +) showed R_{Gly} 0·56 (possibly due to V 1) and a third spot (+ +) showed R_{Val} 0·92. The third spot was violet at first but faded to brown–grey within 36 hr. It was not resolved from spots due to authentic DL-pipecolic acid in system A or B and in the latter showed R_{Val} 1·15. It was coloured green when the papers were sprayed with 0·2% isatin in acetone and heated for 5 min. at 100°. Pipecolic acid yielded a green spot under similar conditions.

(*c*) *Treatment with sodium in liquid ammonia.* The thiazole (20 mg.) was added to liquid NH_3 (5 ml.). Small pieces of

Na were added until a persistent blue colour appeared and the colour was then discharged by addition of a crystal of NH_4Cl. The solution was allowed to evaporate to dryness and the residue was heated in 6N-HCl (10 ml.) for 16 hr. at 110°. The HCl was removed *in vacuo* and the residue examined by electrophoresis on paper at pH 7, followed by chromatography on paper in system A. The material with no net charge at pH 7 showed two spots, one (++) with the same R_F as that of alanine and the other (++) with R_{Gly} 0·56 (possibly due to VI). Two spots due to material which migrated towards the anode corresponded in position with α-aminoadipic acid (++) and unchanged thiazole respectively. A spot (++) due to material which migrated towards the cathode corresponded in position with lysine.

Racemization of the thiazole from cephalosporin C. The thiazole (50 mg.) was dissolved in 0·91N-NaOH (0·4 ml.) Acetic anhydride (0·28 ml.) was added drop by drop with vigorous shaking. The mixture was kept at 37° in a sealed tube for 20 hr. and then evaporated to dryness in a rotary evaporator. The remaining gum was dissolved in water (0·6 ml.) and 0·4 ml. of N-HCl was added. No precipitate formed and the solution was again evaporated to dryness, yielding a gum and crystals of NaCl. The gum was dissolved in acetone–ethanol (about 1:1, v/v) and the clear solution re-evaporated. The amorphous residue was heated in 3N-HCl (2 ml.) for 16 hr. at 105° and the solution evaporated *in vacuo*. The residue was dissolved in water (1 ml.) and added to a column of Dowex 1 (×10) (1 cm. × 25 cm., 200–400 mesh) in the acetate form. Elution was carried out with 0·5N-acetic acid, 5 ml. fractions being collected. A major band of material (as gauged by absorption at 237 mμ) emerged in fractions 21–31. These fractions were evaporated in a rotary evaporator and yielded a crystalline residue. The latter was mixed with water (0·2 ml.) and about 0·4 ml. of aq. 0·4N-NH_3 added, when the crystals dissolved to give a clear solution. 4N-Acetic acid was added until the pH fell to about 3·8. After a short interval the product separated in crystalline form, and was collected, washed with a little water, and dried *in vacuo* at 100° (21 mg.). It showed no detectable optical activity in N-HCl (*c*, 3·7; *l*, 0·5 dm.). It melted at 233–237° and showed $\lambda_{max.}$ at 237 mμ in water (log ε 3·80). It could not be distinguished from the original optically-active thiazole by electrophoresis on paper at pH 7 or chromatography on paper in system A (Found: C, 43·9; H, 5·1; N, 11·7; S, 13·4. $C_9H_{12}O_4N_2S$ requires C, 44·2; H, 4·9; N, 11·5; S, 13·1%.)

Synthesis of 5-acetamido-5:5-diethoxycarbonylvaleronitrile. Diethyl acetamidomalonate (1·37 g.) was dissolved in 2 ml. of ethanol (dried with Mg and freshly distilled) in a Pyrex test-tube (12 cm. × 3 cm. diam.) with a B 24 ground-glass joint. To this solution was added a solution of Na (0·145 g., 1 mol.prop.) in ethanol (1·5 ml.). The resulting solution (protected by a soda-lime tube) was warmed in an oil bath at 60–70° for 15 min., during which time a white precipitate appeared. The mixture was then evaporated *in vacuo* to a syrup, the Pyrex tube being used as the distillation flask of a rotary evaporator. The evaporator was run for a further 2 hr. while the syrup was heated to about 60° by means of an infrared lamp. This led the syrup to crystallize. The solid was scraped from the walls of the tube and γ-chlorobutyronitrile (1·23 g., 1·9 mol.prop.) (L. Light and Co., Poyle, Bucks) was added. An air-cooled reflux condenser was then fitted, and the tube heated in an oil bath at 165–175° so that the γ-chlorobutyronitrile refluxed. After 17 hr., the reaction mixture, which was no longer alkaline, was allowed to cool to room temperature and stirred with dry ethanol (20 ml.). The solution was filtered, and the solid washed twice with 10 ml. portions of boiling ethanol. The filtrates were combined and evaporated *in vacuo* in a rotary evaporator, the flask being finally heated by means of an infrared lamp (to about 60°) so that much of the excess of γ-chlorobutyronitrile was removed. The residue was purified by countercurrent distribution in the system *n*-butyl acetate–water, 15 ml. of upper phase and 20 ml. of lower phase being used and the upper phase being mobile. After 100 fundamental transfers, 45 withdrawals of upper phase were made from tube 100. Dry weights were determined on samples (0·5 ml.), first of every fifth withdrawn fraction, and subsequently of every second fraction in regions close to peaks of material. Some material (possibly unchanged γ-chlorobutyronitrile) was present in the early withdrawn fractions, the weight of this material declining from a maximum in the first withdrawal to zero in the ninth. The main band of material was found in withdrawn fractions 16–35 and reached a maximum in fraction 26. Samples of material from these fractions were hydrolysed by heating in 6N-HCl for 12 hr. at 110°, and the hydrolysates examined by paper electrophoresis in collidine acetate (pH 7) and by paper chromatography in system A, the spots being developed with ninhydrin. All the hydrolysates contained α-aminoadipic acid together with a trace of neutral material, but those from fractions 16–22 contained in addition a small amount of an unidentified amino acid which moved a little faster than α-aminoadipic acid both on electrophoresis and chromatography. The occurrence of this material correlated closely with a small hump in the leading edge of the main band of the dry-weight curve. Fractions 23–32 were therefore combined and evaporated to dryness *in vacuo* in a rotary evaporator. The syrup so obtained crystallized during the course of the next week, and was recrystallized from *cyclo*hexane as colourless needles, m.p. 51–52° (0·5 g., 28%) (Found: C, 54·8; H, 7·5; N, 9·8. $C_{13}H_{20}O_5N_2$ requires C, 54·9; H, 7·1; N, 9·9%).

Synthesis of 5:5-diethoxycarbonyl-5-phthalimidovalerothioamide (thioamide A). Diethyl phthalimidomalonate was prepared according to the procedure recommended by Osterberg (1944) and converted by the method of Sörensen (1903–6) into 5:5-diethoxycarbonyl-5-phthalimidovaleronitrile, which was recrystallized from di*iso*propyl ether.

Hydrogen sulphide, generated in a Kipps apparatus from FeS and 4N-HCl, was freed from acid spray by bubbling through water, dried by passage through a $CaCl_2$ tower, and condensed by passing into a Pyrex tube cooled to −70° in a mixture of ethanol and solid CO_2. Liquid H_2S required for the reaction was then transferred as required to the reaction vessel, connected to this reservoir, by removing the cold bath and replacing it around the reaction vessel.

Into 5:5-diethoxycarbonyl-5-phthalimidovaleronitrile (1·92 g.) and triethylamine (about 0·2 ml.) was condensed liquid H_2S (15 ml.). The Pyrex tube containing this mixture was quickly transferred to a stainless-steel bomb, which was sealed and immersed in water at 75° in a lagged polythene bucket. The rate of cooling was such that the water temperature fell to 45° in 5 hr. The bomb was kept in the bucket for a total of 50 hr. The pressure was then released, excess of H_2S allowed to evaporate, and residual volatile

material was removed from the syrupy product by warming to 70° under water-pump vacuum. The residue was dissolved in benzene (about 40 ml.), washed with 0·2M-KH_2PO_4 (about 20 ml.) and then with water, and the benzene solution evaporated to dryness in a rotary evaporator. The syrup so obtained was purified by countercurrent distribution in the system methanol–benzene–water (1:1:1, by vol.), 20 ml. of lower layer and 19 ml. of upper layer being used in each tube and the upper layer being mobile. After 100 fundamental transfers, 70 withdrawals were made from the 101st tube. Samples (2 ml.) were taken from every fifth withdrawn fraction and dry weights determined. Further samples were then taken in the neighbourhood of dry-weight peaks. Dry weight was negligible in the withdrawn fractions beyond the fiftieth, the main peak lying in the twelfth withdrawn fraction ($K = 8$), and a very small peak occurring in the region of the thirty-seventh withdrawal ($K = 2·7$). Withdrawn fractions 8–17 were combined and evaporated to dryness in a rotary evaporator, giving a syrup (approximately 500 mg., yield 25%) which crystallized during the course of the next few days. Recrystallization from CCl_4 and a little $CHCl_3$ gave pale ivory-coloured crystals, m.p. 143–145°, raised on recrystallization from ethanol–water to 144–145°. The infrared spectrum of the compound ($CHCl_3$ solution) showed peaks at 1785 and 1722 cm.$^{-1}$, consistent with the presence of the phthalimide ring (Sheehan *et al.* 1953). The ultraviolet-absorption spectrum (ethanol solution) showed a maximum at 220 mμ (log ε 4·62), in which it resembled that of the parent nitrile ($\lambda_{max.}$ 220 mμ, log ε 4·6) but differed from that of phthalic acid and dimethylphthalate, which show a weaker maximum at 225 mμ (log ε = 3·87). A second maximum at 269 mμ (log ε 4·02) was consistent with the presence of the thioamide group (Burawoy, 1939). The compound thus appeared to be 5:5-*diethoxycarbonyl*-5-*phthalimidovalerothioamide* (Found: C, 56·1; H, 5·7; N, 7·2; S, 8·0. $C_{19}H_{22}O_6N_2S$ requires C, 56·1; H, 5·5; N, 6·9; S, 7·9%).

Synthesis of thioamide B. In a previous experiment, 5:5-diethoxycarbonyl-5-phthalimidovaleronitrile (1·5 g.) was similarly treated with liquid H_2S in a bomb for 14 hr. The product was freed from volatile material as described above, but the syrup was then subjected to countercurrent distribution in the system benzene–methanol–water (1:1:1, by vol.) without first being dissolved in benzene and washed with buffer solution. In this case, only 24 fundamental transfers were carried out, and 20 withdrawals made from tube 24. Analysis by dry weight revealed a band in the first four withdrawn fractions. The first fraction was found to contain largely unchanged nitrile. The following three fractions were not worked up, but presumably contained thioamide A. Overlapping the first withdrawn band was a broad band with peak in the region of the seventh withdrawal ($K = 3$). The seventh to twelfth withdrawn fractions were combined and evaporated in a rotary evaporator. The syrup so obtained crystallized on standing, and was easily recrystallized from a little ethanol at −10°, giving needles, m.p. 124–126° (500 mg., 30%). After a second recrystallization from a mixture of $CHCl_3$, CCl_4, and a little hexane the product (thioamide B) melted at 123·5–124·5°. Its infrared spectrum ($CHCl_3$ solution) showed bands at 1671 and 1578 cm.$^{-1}$, consistent with the presence of a secondary amide group and a band at 1735 cm.$^{-1}$ which could be attributed to ester groups. The absence of bands at 1785 and 1722 cm.$^{-1}$ indicated that the phthalimide ring was not present. The ultraviolet spectrum of the product (ethanol solution) showed a plateau at 220–225 mμ resembling the broad maximum at 225 mμ (log ε 3·86) shown by phthalic acid and its dimethyl ester. The presence of the thioamide group was indicated by a maximum at 270 mμ (log ε 4·02) (cf. Burawoy, 1939). This evidence seemed consistent with the structure 5-(*o*-carboxybenzamido)-5:5-diethoxycarbonylvalerothioamide for thioamide B (Found: C, 54·2; H, 6·1; N, 6·6; S, 7·7. $C_{19}H_{24}O_7N_2S$ requires C, 53·7; H, 5·7; N, 6·6; S, 7·6%). However, the compound contained no group which could be titrated in 80% (v/v) ethanol between pH 2 and pH 11, and attempts to precipitate salts from an ethereal solution of the compound by addition of anhydrous bases were unsuccessful. The structure of thioamide B thus remains uncertain.

Synthesis of 2-(4-amino-4-carboxybutyl)thiazole-4-carboxylic acid. 1. *From 5:5-diethoxycarbonyl-5-phthalimidovaleronitrile without isolation of the thioamide.* The nitrile (300 mg.) was treated with liquid H_2S and a catalytic amount of triethylamine, as described above. Volatile material was removed by warming under water-pump vacuum, and to the syrupy residue was added a solution of methyl bromopyruvate (approx. 200 mg.) (Kuhn & Dury, 1951) in dry ethanol (2 ml.). The mixture was warmed to 70° for 1 hr., and allowed to stand at room temperature overnight. The ethanol was evaporated in a stream of air, the residue dissolved in acetic acid (0·5 ml.) and the solution transferred to a small Pyrex tube. After addition of an equal volume of 11N-HCl, the tube was sealed and heated at 110° for 16 hr. The solvent was evaporated by heating at 100° in a stream of air. The residue was dissolved in 0·5N-acetic acid (2 ml.) and applied to a column (26 cm. × 1 cm. diam.) of Dowex 1 (×10) (acetate form, 200–400 mesh). Elution was carried out with 0·5N-acetic acid, thirty-nine 2 ml. fractions and then thirty-two 5 ml. fractions being collected. Every second fraction was examined by ninhydrin reaction and by absorption at 237 mμ. A sharp band of ninhydrin-positive material (which behaved as α-aminoadipic acid when examined by electrophoresis and chromatography on paper) emerged in fractions 4–15. Fractions 41–54 contained the only other ninhydrin-positive band, and showed absorption at 237 mμ. Fractions 43–52 were combined and evaporated to dryness in a rotary evaporator. The solid so obtained was dissolved in water (0·5 ml.) by the dropwise addition of aq. N-NH_3 solution (to pH 7·5) and a few undissolved particles were removed by centrifuging. The thiazole was then recrystallized by adjusting the solution to pH 3·8 with 2N-acetic acid. Yield, 93 mg. (47%). The thiazole had m.p. 235–237° (decomp.); $\lambda_{max.}$ (water) 237 mμ (log ε 3·79); $\lambda_{max.}$ (N-HCl) 233 mμ, (log ε 3·91). The change in $\lambda_{max.}$ and $\epsilon_{max.}$ occurs gradually over the range 0·5–0·2N-HCl (Found: C, 43·9; H, 4·9; N, 11·1; S, 12·6. $C_9H_{12}O_4N_2S$ requires C, 44·2; H, 4·9; N, 11·4; S, 13·1%).

2. *From 5-acetamido-5:5-diethoxycarbonylvaleronitrile without isolation of the thioamide.* To the nitrile (32 mg.) was added a drop of triethylamine, and approximately 5 ml. of liquid H_2S, and the mixture was heated in a bomb for 14 hr., according to the procedure described above. The product was freed from volatile material, and condensed with methyl bromopyruvate (approximately 100 mg.), and the condensation products were hydrolysed, as in the previous experiment. Electrophoresis on paper at pH 7·0 and chromatography on paper in system A indicated that

the hydrolysate contained the required thiazole (indistinguishable from the product obtained in the previous experiment, and from that obtained from cephalosporin C) together with an almost equal amount of α-aminoadipic acid.

3. *From thioamide A.* Thioamide A (9·3 mg.) was condensed with methyl bromopyruvate (56 mg.) in ethanol solution (0·5 ml.), and the products were hydrolysed as described above. The hydrolysate was evaporated to dryness and the residue examined by chromatography on paper in system A and electrophoresis on paper at pH 7·0. This indicated that the residue consisted of the required thiazole and a trace of α-aminoadipic acid. Comparison of the intensity of the spot (coloured with ninhydrin) corresponding to the thiazole with the intensities of spots obtained with known amounts of the thiazole from cephalosporin C indicated that the synthetic thiazole had been formed in almost quantitative yield.

4. *From the thioamide B.* Thioamide B (9·3 mg.) and methyl bromopyruvate (7 mg.) were condensed in ethanol solution (0·5 ml.) and the products hydrolysed in the manner described above. After evaporation of the hydrolysate to dryness, the residue was examined by electrophoresis on paper at pH 7·0 and chromatography on paper in system A. This indicated that the residue consisted of the required thiazole and a trace of α-aminoadipic acid. Comparison of the intensity of the ninhydrin colour with that given by known concentrations of the thiazole isolated from cephalosporin C indicated that the synthetic thiazole had been formed in almost quantitative yield.

Behaviour on paper chromatography of 2-(4-amino-4-carboxybutyl)thiazole-4-carboxylic acid. The synthetic thiazole and the thiazole from cephalosporin C were indistinguishable on paper chromatography in systems A, B, C, D and E. The following R_{Gly} values were obtained in systems A–D: A, 1·76; B, 1·37; C, 3·5; D, 0·56. In system E R_{Gly} was 2·12 when the specimen was applied to the paper in N-HCl and 0·73 when it was applied in aq. 3% (w/v) NH_3, glycine being applied in neutral solution in each case.

SUMMARY

1. A mixture of acidic, neutral and basic products was formed when cephalosporin C was subjected to mild hydrolysis in hot dilute acid. Oxidation of the neutral material with silver oxide yielded an acidic product which behaved like authentic δ-amino-δ-carboxyvalerylglycine on paper chromatography or electrophoresis, and which gave α-aminoadipic acid and glycine on hydrolysis with N-hydrochloric acid at 110°. δ-Amino-δ-carboxyvalerylglycine had previously been obtained from cephalosporin N [(D-4-amino-4-carboxybutyl)penicillin] under similar conditions.

2. An acidic compound, formed when cephalosporin C was kept in neutral aqueous solution at 37°, was isolated by chromatography on an anion-exchange resin. The physical and chemical properties of this compound, including its behaviour on hydrogenolysis, suggested that it was 2-(D-4-amino-4-carboxybutyl)thiazole-4-carboxylic acid. The compound was racemized by treatment with acetic anhydride in aqueous solution.

3. 2-(DL-4-Amino-4-carboxybutyl)thiazole-4-carboxylic acid was synthesized from methyl bromopyruvate and either 5:5-diethoxycarbonyl-5-phthalimidovalerothioamide or the corresponding acetamido compound, the thioamides being prepared from the corresponding nitriles. The synthetic compound was shown to be identical with the racemized product from cephalosporin C.

4. Some implications of these findings are discussed.

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REFERENCES

Abraham, E. P. & Newton, G. G. F. (1954). *Biochem. J.* **58**, 266.

Abraham, E. P. & Newton, G. G. F. (1956). *Biochem. J.* **62**, 658.

Abraham, E. P. & Newton, G. G. F. (1958). In *Amino Acids and Peptides with Antimetabolic Activity.* London: J. and A. Churchill Ltd.

Brookes, P., Clark, R. J., Majhofer, B., Mijović, M. P. V. & Walker, J. (1960). *J. chem. Soc.* p. 925.

Brookes, P., Fuller, A. T. & Walker, J. (1957). *J. chem. Soc.* p. 689.

Burawoy, A. (1939). *J. chem. Soc.* p. 1177.

Craig, L. C., Gregory, J. D. & Hausmann, W. (1950). *Analyt. Chem.* **22**, 1462.

Dent, C. E. (1948). *Biochem. J.* **43**, 169.

du Vigneaud, V. & Meyer, C. E. (1932). *J. biol. Chem.* **98**, 295.

Erne, M. & Erlenmeyer, H. (1948). *Helv. chim. acta*, **31**, 652.

Felix, A. & Pitt, R. M. (1935). *J. Hyg., Camb.*, **35**, 428.

Grobbelaar, N., Zacharius, R. M. & Steward, F. C. (1954). *J. Amer. chem. Soc.* **76**, 2912.

Hale, C. W., Abraham, E. P. & Newton, G. G. F. (1958). *Int. Abst. biol. Sci.* Suppl. 1–68.

Kuhn, R. & Dury, K. (1951). *Liebigs Ann.* **571**, 44.

Lockhart, I. M., Abraham, E. P. & Newton, G. G. F. (1955). *Biochem. J.* **61**, 534.

Newton, G. G. F. & Abraham, E. P. (1954). *Biochem. J.* **58**, 103.

Osterberg, A. E. (1944). *Org. Synth.* (Coll.) **1**, 271.

Pavlic, A. A. & Adkins, H. (1946). *J. Amer. chem. Soc.* **68**, 1471.

Sheehan, J. C., Henery-Logan, K. R. & Johnson, D. R. (1953). *J. Amer. chem. Soc.* **75**, 3292.

Sörensen, S. L. P. (1903–06). *C.R. Lab. Carlsberg*, **6**, 22.

Woiwod, A. J. (1949). *J. gen. Microbiol.* **3**, 312.

Wright, B. E. & Stadtman, T. C. (1956). *J. biol. Chem.* **219**, 863.

Biochem. J. (1960) **75**, 224

Tissue Components of the Domestic Fowl

4. PLASMA-ALKALINE-PHOSPHATASE ACTIVITY*

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The alkaline-phosphatase activity of the plasma (or serum) of the domestic fowl has been the subject of a number of investigations by various methods of assay (Auchinachie & Emslie, 1934; Common, 1934, 1936; Peterson & Parrish, 1939*a*, *b*; Wiese, Johnson, Elvehjem, Hart & Halpin, 1939; Motzok, 1950*a*, *b*, 1959; Stutts, Briles & Kunkel, 1957). In general, single measurements were made on individual birds although occasionally subsequent measurements were made on the same animal; however, Common (1936) records a number of experiments where white Wyandotte pullets and cockerels were examined at approximately monthly intervals. There is agreement that the plasma activities in cocks and in immature birds show less variability between individuals than do the activities in hens and that no significant differences could be demonstrated between hens when 'in' or 'out of lay'. There are no records of measurements more frequent than monthly on individual birds during a lengthy period of observation.

The plasma enzyme is assumed to have its origin in the bones, and to be associated with osteoblastic activity (Hall & King, 1930–31; Franseen, Simmons & McLean, 1939; Woodard & Higinbotham, 1941; Cade, Maclagan & Townsend, 1940; and histological observations in this Laboratory). During the investigation at this Centre of an outbreak of 'cage-layer fatigue' (cf. Couch, 1955; Francis, 1957) it became necessary to study the plasma-alkaline-phosphatase activity in healthy birds, since Bell, Siller & Campbell (1959) had found that the syndrome in question involves a bone dystrophy.

This paper describes a simple spectrophotometric micromethod to measure the enzymic hydrolysis of 4-nitrophenyl phosphate and includes observations made at frequent intervals over 6 months on a small group of hens kept in the same environment.

METHODS

The birds examined were brown Leghorns of the flock maintained on the standard diet (Bell, 1957). Blood samples were taken from the wing vein into a heparinized syringe and the plasma was separated as soon as possible after bleeding. In agreement with Motzok & Wynne (1950), the alkaline-phosphatase activity of fowl plasma against both 4-nitrophenyl phosphate and glycerol 2-phosphate was not found to alter on keeping the plasma for 2 weeks in the refrigerator.

Alkaline-phosphatase activity of plasma

4-Nitrophenyl phosphate was first used by Ohmori (1937) as a substrate in visual colorimetric measurements of enzymic activity; later, Bessey, Lowry & Brock (1946) used this substrate in a micro-assay on human blood which employed but 5 μl. of plasma. The simplified method described below owes much to these authors and to the work of J. D. Bell (1955).

Reagents. The buffer (Delory & King, 1945) consisted of 0·1 M-Na_2CO_3–0·1 M-$NaHCO_3$ (7:3), pH 10·08 at 37°. The substrate consisted of 0·066 M-disodium 4-nitrophenyl-phosphate (L. Light and Co. Ltd.) recrystallized according to Ohmori (1937). Free nitrophenol was removed from the solution by threefold extraction with water-saturated butan-1-ol followed by extraction with moist ether; dissolved ether was removed by a stream of moist air. Although the solution was stored in the refrigerator some slight hydrolysis of the ester took place but this was not of consequence since the blanks contained substrate equal to that used in the digests. The standards were prepared from a stock aqueous solution of purified 4-nitrophenol (200 μg./ml.) by dilution with 0·05 M-NaOH to concentrations (μg./ml.) of 0·25, 0·50, 1·25, 2·50, 5·0, 10·0. With a 1 cm. light path these solutions gave a linear graph when measured at 397 mμ in the Unicam SP. 500 spectrophotometer. (Other authors have recorded their measurements at 400 mμ; on our instrument a definite maximum was found at 397 mμ.)

Activity measurements. Owing to the wide variations between plasma samples in colour and turbidity it was found necessary to run a plasma blank with each digest. In this way considerable colours in the sample, even visible amounts of haemoglobin, are compensated for.

Buffer (1 ml.) was placed in suitable vessels, e.g. test tubes 15 cm. × 1·6 cm., followed by plasma (0·05 ml.); after mixing at room temperature (about 15°) substrate (0·1 ml.) was quickly added and mixed with the contents of each vessel, all of which were then kept at 37° for 60 min. (although the body temperature of the fowl is 41·5°, the former temperature was used as it is customary for laboratory incubation). At the end of the incubation period the mixture in each vessel was diluted with a measured volume of 0·05 M-NaOH; according to the enzymic activity of the plasma the amount of diluent added may vary between 3 and 25 ml. in order to obtain a final concentration of 4-nitrophenol of the order of 2–7 μg./ml. The method has thus considerable flexibility.

* Part 3: Bell, McIndoe & Gross (1959).

Table 1. *Comparison of Bodansky, King–Armstrong and 4-nitrophenol assays of alkaline-phosphatase activity in plasmas of hens*

Ratios are calculated on the basis of the inorganic phosphate liberated from each substrate in 60 min. at 37° in 0·1 M-Na_2CO_3–$NaHCO_3$ buffer, pH 10·08, by 100 ml. of plasma. Activities are expressed in the conventional 'units'.

Date of assay	No. of hen	Activities: Bodansky (B)	Activities: King–Armstrong (KA)	Activities: 4-Nitrophenol (NP)	Ratios: NP/B	Ratios: NP/KA	Ratios: KA/B
19. ii. 59	1	13·6	149	118·0	1·9	0·54	3·6
	8	3·0	31·8	27·0	2·0	0·57	3·5
23. ii. 59	8	—	20·3	18·0	—	0·61	—
26. ii. 59	1	3·2	37·4	31·4	2·2	0·57	3·8
	2	2·9	30·7	25·8	2·0	0·57	3·5
	3	1·8	15·4	15·4	1·9	0·67	2·8
	4	3·4	31·3	28·9	1·9	0·63	3·4
	5	—	36·7	35·7	—	0·66	—
	6	—	27·4	24·0	—	0·59	—
3. iii. 59	1	—	32·0	25·8	—	0·55	—
	2	—	50·0	53·5	—	0·72	—
	3	—	29·5	31·4	—	0·71	—
	4	—	26·0	22·0	—	0·59	—
	5	—	36·5	29·5	—	0·55	—
	6	—	40·5	36·7	—	0·64	—
	8	—	22·5	20·0	—	0·63	—
				Means	1·99	0·61	3·4

A blank was set up for each plasma sample and incubated with the digests; the blank contained buffer (1 ml.) and plasma (0·05 ml.). At the end of the incubation, 0·05 M-NaOH was added in amount equal to that added to the digest. After mixing, substrate (0·1 ml.) was added and the whole mixed. Each diluted digest was measured at 397 mμ against its blank and the 4-nitrophenol content obtained from the standard curve.

Excellent agreement between duplicates was obtained; furthermore, if the solutions were kept in the refrigerator no change in the difference between digest and blank could be found after 24 hr.

Activity. This is expressed as mg. of 4-nitrophenol liberated by 100 ml. of plasma in 60 min. at 37° with the buffer and substrate mentioned above.

RESULTS

A comparison of three methods of assay of plasma-alkaline-phosphatase activity. The 4-nitrophenyl phosphate method was compared with assays by the methods of Bodansky (after Hawk, Oser & Summerson, 1947) and King & Armstrong (1934); the latter estimations were done independently by Mr T. P. Bird. In all estimations the same buffer and period of incubation were used; the results are given in Table 1. Except for this experiment all measurements described below were made by the nitrophenyl phosphate method.

As stated in Table 1, the ratios between the activities measured by the three methods are assessed from the amounts of phosphate liberated from three different esters under the same conditions of incubation; the conventional concentrations of glycerol 2-phosphate and phenyl phosphate were used. Although it is well known that it is not easy to obtain close agreement between measurements of alkaline-phosphatase activities against different substrates, each of the three ratios (nitrophenyl phosphate method/Bodansky method, nitrophenyl phosphate method/King–Armstrong method and King–Armstrong method/Bodansky method) varies between reasonably narrow limits. These variations may be due either to errors of manipulation or to the alkaline phosphatase of the plasma consisting of a mixed and variable population of enzymes having different activities against the different substrates. The ratios are sufficiently constant to enable the orders of the results of others, by any of the three methods, to be assessed in terms of either the Bodansky, the King–Armstrong or the nitrophenyl phosphate method.

A survey of plasma-alkaline-phosphatase activity in six hens in different physiological states. Six hens (nos. 1–6) chosen for high laying rate and in their second year of production, were examined at frequent intervals from 21 November 1958 (when they were in moult) until 29 May 1959; in all, 75 measurements were made on each of five birds and 58 on the sixth. The birds were caged in a well-ventilated room and, when in lay, received light for 14 hr./24 hr.; the temperature of the room was not controlled, but the birds were not subjected to the vagaries of temperature experienced in the general animal house. The findings are given in Tables 2 and 3.

Table 2 is an attempt to represent, in a condensed form, a large number of observations. It is clear that the activity of the plasma enzyme in one hen can be very different from that in another; it therefore is illogical to attempt to derive mean figures for the groups in the different physiological states. During the moulting and post-moulting non-laying periods each bird maintained a relatively steady level of activity, as indicated from the S.D. values. The first 2 months of laying were marked by large fluctuations in the plasma-alkaline-phosphatase activity; the fluctuation in activity decreased very markedly during the subsequent months in four birds whereas the remaining two continued to show rises and falls of the same order as they did when coming into lay. In all these hens, the lowest levels of enzymic activity were of much the same order whatever the physiological state. Since the metabolism of the heavily laying hen is in a state of constant flux it is surprising that any agreement at the lower levels of enzymic activity can be observed at all without taking precautions to obtain the plasma at identical times during the laying cycle.

The results expressed in Table 3 amplify those of Table 2. Hens nos. 1, 2 and 6 appear relatively homeostatic whereas nos. 3 and 4 show wide day-to-day fluctuation.

Cocks and immature birds. Six adult cocks were observed for 5 successive days (Table 4) and a few single measurements were made on immature birds of both sexes and some repetitions on the females (Table 5).

The cocks, which were kept in the same pen, showed quite characteristic individual levels with little fluctuation; the values found for plasma-

Table 2. *Alkaline-phosphatase activity in plasma of six hens in three physiological states*

Condition	Hen no.	Period covered by observations (dates inclusive)	No. of observations	Plasma-phosphatase activity				Eggs laid
				Highest	Lowest	Mean	S.D.	
Moulting and refeathering	1	21. xi.–8. xii. 58	8	97	39	64	18·2	—
	2	21. xi.–1. xii. 58	5	56	29	43	10·9	—
	3	21. xi.–18. xii. 58	11	60	26	42	12·0	—
	4	21. xi.–12. xii. 58	10	36	18	28	6·4	—
	5	21. xi.–10. xii. 58	9	56	31	41	9·4	—
	6	21. xi.–28. xi. 58	4	83	56	71	13·4	—
Non-laying (post-moult)	1	10. xii. 58–27. i. 59	16	112	43	82	19·1	—
	2	3. xii. 58–26. xii. 58	8	60	32	53	12·5	—
	3	22. xii. 58–30. i. 59	16	68	24	39	11·3	—
	4	18. xii. 58–2. i. 59	6	92	27	52	22·0	—
	5	12. xii. 58–29. xii. 58	5	68	39	53	10·5	—
	6	12. xii. 58–19. i. 59	13	116	43	64	18·6	—
Laying, first 28 days	1	27. i. 59–23. ii. 59	8	300	42	125	87·1	20
	2	28. xii. 58–24. i. 59	10	135	22	49	32·6	12
	3	2. ii. 59–1. iii. 59	7	186	15	73	68·0	16
	4	5. i. 59–1. ii. 59	9	166	29	75	55·6	16
	5	30. xii. 58–26. i. 59	11	175	31	80	47·7	18
	6	21. i. 59–17. ii. 59	8	184	37	99	50·5	18
Second 28 days	1	24. ii.–23. iii.	8	133	26	55	36·0	24
	2	25. i.–21. ii.	7	160	45	102	43·4	17
	3	2. iii.–29. iii.	8	93	18	44	25·7	22
	4	2. ii.–1. iii.	7	280	29	124	85·5	16
	5	27. i.–23. ii.	7	166	41	100	46·5	7*
	6	18. ii.–17. iii.	8	109	24	62	28·7	20
Third 28 days	1	24. iii.–20. iv.	15	61	28	40	10·2	23
	2	22. ii.–21. iii.	8	55	18	40	13·5	16
	3	30. iii.–26. iv.	15	55	17	35	8·4	22
	4	2. iii.–29. iii.	8	152	25	77	55·2	20
	5	24. ii.–23. iii.	8	87	30	47	19·3	8*
	6	18. iii.–14. iv.	14	157	26	58	37·4	20
Fourth 28 days	1	21. iv.–18. v.	11	58	20	40	12·9	23
	2	22. iii.–18. iv.	15	69	20	37	16·4	16
	3	26. iv.–24. v.	13	145	28	78	33·3	18
	4	30. iii.–26. iv.	18	210	29	86	56·5	22
	5	24. iii.–22. iv.	15	72	21	47	14·1	13*
	6	15. iv.–12. v.	13	72	25	46	13·2	21
Fifth 28 days	2	19. iv.–16. v.	11	55	30	42	8·82	22
	4	27. iv.–24. v.	13	290	65	130	60·30	22

* This hen was killed on 22. iv. 59 for post-mortem examination. It had been 'laying internally', i.e. the yolks had passed into the peritoneal cavity and therefore no shells had been formed on many potential eggs.

alkaline-phosphatase activity were of the same order as the lowest levels of the non-laying and laying hens (Table 2).

The immature birds (5–6 weeks old) showed high values, as might be expected in rapidly growing animals. At 8½–10 weeks, however, it will be seen that many of both sexes had plasma enzymic activities more of the order of those found in adult hens, although rapid growth of these birds continues for some weeks at apparently the same rate as the 5–6-week-old animals.

Table 3. *Groups of daily measurements of plasma-alkaline-phosphatase activity on five laying hens in spring* 1959

Periods when measurements were made; A, 1–3. iv.; B, 6–10. iv.; C, 13–17. iv.; D, 27. iv.–1. v.; D, 4–8. v.; F, 25–29. v.

	Plasma-alkaline-phosphatase activity				
Period	Hen 1	Hen 2	Hen 3	Hen 4	Hen 6
A	33	20	17	29	42
	32	28	40	38	85
	54	29	44	75	157
B	42	40	40	58	46
	34	23	20	32	45
	36	27	23	41	47
	36	36	37	49	41
	41	41	26	120	62
C	28	31	41	41	31
	22	28	38	81	28
	27	45	32	120	51
	20	63	40	210	44
	38	69	40	135	34
D	28	55	55	74	37
	22	38	45	65	37
	27	34	47	101	25
	20	34	94	153	37
	38	43	145	290	63
E	48	44	71	82	46
	47	30	62	88	50
	44	47	97	105	60
	58	46	72	125	53
	57	52	49	130	72
F	56	116	43	195	41*
	75	92	28	220	57
	58	85	33	135	43
	61	128	59	168	38
	77	117	58	115	40

* Hen no. 6 was out of lay between 12. v. and 30. v.

Table 4. *Daily plasma-alkaline-phosphatase activity of six adult cocks*

	Consecutive days					
Cock	1	2	3	4	5	Mean
A	37	23	20	24	23	25
B	57	42	41	42	30	40
C	65	42	41	48	49	59
D	81	62	61	71	79	71
E	47	33	33	39	38	38
F	32	20	23	26	30	26

DISCUSSION

Throughout the following discussion it must be borne in mind that the plasma alkaline phosphatase of the healthy fowl is understood to originate in the bones, and particularly in the osteoblasts; the enzymic activity of the plasma therefore reflects the activity of a mechanism for skeletal calcification (cf. Hall & King, 1930–31; Auchinachie & Emslie, 1934; Common, 1934, 1936; Motzok, 1950*a*, *b*). Egg-shell calcification, on the other hand, is mediated by carbonic anhydrase in the shell gland (Common, 1941; Gutowska & Mitchell, 1945) and therefore is not directly dependent on any mechanism in which alkaline phosphatase is concerned. Indirectly, however, a high proportion of the shell requirements have to be met from skeletal sources; Driggers & Comar (1949) and Jowsey, Berlie, Spinks & O'Neil (1956) have demonstrated, with ^{45}Ca, that food in the gut supplies rather less than half of shell calcium. The bones have long been known to be a source of shell calcium; in the immediate pre-laying weeks, increased mineralization of the skeleton can be observed; in eight of ten hens observed in the present series a definite peak in plasma-alkaline-phosphatase activity was noted to precede the onset of lay.

There must be a considerable turnover of skeletal calcium (cf. Winget & Smith, 1958; Winget, Smith & Hoover, 1958) in the laying hen and the very active recalcification concerned would be expected to cause an increase in plasma phosphatase, as indeed was observed during the early months after the onset of lay (Table 2). Despite the maintenance of a high rate of production, in three hens (nos. 1, 2 and 6) the level of enzymic activity in the plasma became less and less variable as time went on. In nos. 3 and 4, on the other hand, variability remained high throughout the months of observation (Table 3). The behaviour of no. 5, which was found to be 'laying internally', is of some interest as this hen produced relatively few shelled eggs although it was known to have active ovaries, from the continued presence in its plasma of the characteristic 'lipophosphoprotein complex' (McIndoe, 1957, 1959). The fall in variability in plasma-alkaline-phosphatase activity in this bird resembles that of nos. 1, 2 and 6.

An explanation of the differences between the behaviour of the different birds is not obvious, but several suggestions may be offered. Homeostasis with respect to the mechanisms affecting calcium in circulation between the gut, the skeleton and the oviduct may have become established in nos. 1, 2 and 6, whereas in nos. 3 and 4 a periodical and repetitive osteoblastosis alone may have caused the continued violent fluctuations in plasma

Table 5. *Plasma-alkaline-phosphatase activity in immature fowls*

Figures in parentheses refer to assays on the same bird made 4 days later.

Age (weeks)	No. of birds	Sex	Plasma activity
5–6	11	M.	404, (406); 442, (442); 362, (402); 292, (298); 276, (202); 432 (484); 290; 480; 510; 340; 330
	11	F.	260 (210); 360, (380); 250, (280); 280, (280); 330 (350); 180 (160); 260; 520; 350; 150; 340
8½–10	9	M.	280; 99; 120; 320; 170; 85; 157; 210; 110
	8	F.	99; 85; 170; 160; 150; 126; 510; 120

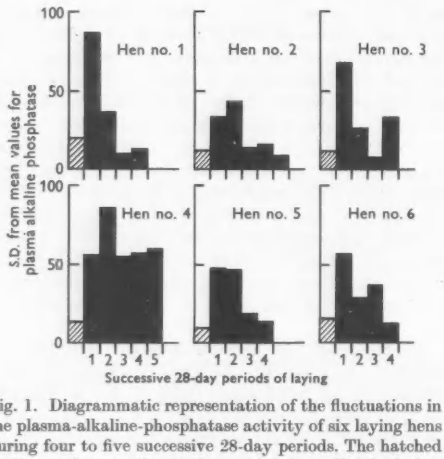

Fig. 1. Diagrammatic representation of the fluctuations in the plasma-alkaline-phosphatase activity of six laying hens during four to five successive 28-day periods. The hatched portions relate to the 28-day period immediately before onset of lay. (For actual levels of activity see Table 2.)

phosphatase. If, as in mammals, plasma alkaline phosphatase is eliminated via the bile, a flexibility in the excretory mechanism of the liver may have developed in nos. 1, 2 and 6 but not in nos. 3 and 4. A third alternative lies in the possibility that improved intestinal absorption, or a decreased excretion, of calcium develops in some birds but not in others, thus sparing transfer of skeletal calcium to the shell.

That individual birds can possess differing biochemical features is illustrated by Tables 2 and 3. Such variabilities in alkaline-phosphatase activity are to be found that an isolated measurement will seldom provide information of value, especially in the laying hen. Common (1936) found, in white Wyandotte pullets, distinct evidence of considerable temporary fluctuations after the onset of lay although his measurements were made at approximately monthly intervals. One of Common's birds showed a remarkably high level of activity comparable with that found by Bell, Siller & Campbell (1959) in cage-layer fatigue. Daily fluctuations can be considerable, as seen from Table 3 (nos. 3 and 4). Bloom, Domm, Nalbandov & Bloom (1958) have shown that, in hens with a good laying record, the medullary bone undergoes sequences of resorption and recalcification concomitant with the formation of the shell. During early stages of shell calcification the medullary bone is rich in both osteoblasts and osteoclasts; as calcification becomes advanced osteoblasts become scarce and osteoclasis is pronounced. During the interval between laying and the next ovulation the numbers of both osteoblasts and osteoclasts in the medullary bone are very variable. If indeed the osteoblasts are the source of plasma alkaline phosphatase, variations in the activity of these cells in the medullary bone may account for some of the fluctuations noted in the present series of experiments.

For comparison purposes, a few daily observations were made on adult cocks (Table 4). Although the activity remained within narrow limits for an individual bird, those limits show a scatter between 25 and 71 units; immature birds of 5–6 weeks (Table 5) have very high activity, as might be expected in growing animals. The values found in a few young birds of 8½–10 weeks show irregularity and some values approach the adult level; Dr J. G. Campbell (unpublished work) has obtained other evidence of rapid fluctuations in the oestrogen–androgen balance of the 6–12-week-old bird. Exogenous sex hormones administered to such immature birds have not yet been shown to influence the plasma-alkaline-phosphatase activity.

It is concluded that egg-shell formation in heavy layers is accompanied by elevated osteoblastic activity, as evinced by an increased fluctuation in the level of the plasma-alkaline-phosphatase activity during the first month after the onset of lay (Fig. 1). Birds which continue to lay eggs with normal shells, at a high rate, may show persistent fluctuations in plasma phosphatase, whereas others become, in some way not yet understood, stabilized with respect to the level of enzyme in their plasma. Isolated analyses on individual birds can easily lead to false conclusions.

SUMMARY

1. A simple and flexible method is described for assessing plasma-alkaline-phosphatase activity against 4-nitrophenyl phosphate. It has been compared with the Bodansky and the King–Armstrong procedures.

2. Both adult non-laying hens and cocks show enzymic activity of the same order. The plasmas of immature birds (5–6 weeks old) of both sexes have alkaline-phosphatase levels up to ten times those found in non-laying adults. At 8½–10 weeks of age great irregularities may be found in that the plasma-alkaline-phosphatase activity of an individual may have dropped to a level near that of the adults, although these birds continue to grow at the same rate as those of 5–6 weeks.

3. In the hen, the average level of plasma enzymic activity is increased by about 50 % upon the bird coming into lay, when wide fluctuations become apparent. The lowest levels in the laying hen are, however, of the same order as those found in the non-laying bird and in the cock.

4. In some hens a fairly steady level of activity with small fluctuations is attained after the first 2 months of intensive egg production. Other birds, however, continue to show very large fluctuations in alkaline-phosphatase activity with no apparent tendency to the establishment of homeostasis with respect to this enzyme.

5. The massive temporary increases in plasma phosphatase shown by the laying hen are believed to reflect a stimulation of the osteoblastic recalcification process called into action by the drain on skeletal calcium required for egg shells.

REFERENCES

Auchinachie, D. W. & Emslie, A. R. G. (1934). *Biochem. J.* **28**, 1993.

Bell, D. J. (1957). *Biochem. J.* **66**, 137.

Bell, D. J., McIndoe, W. M. & Gross, D. (1959). *Biochem. J.* **71**, 355.

Bell, D. J., Siller, W. G. & Campbell, J. G. (1959). *Biochem. J.* **72**, 32P.

Bell, J. D. (1955). Ph.D. Thesis: University of Cambridge.

Bessey, O. A., Lowry, O. H. & Brock, M. J. (1946). *J. biol. Chem.* **164**, 321.

Bloom, M. A., Domm, L. V., Nalbandov, A. V. & Bloom, W. (1958). *Amer. J. Anat.* **102**, 411.

Cade, S., Maclagan, N. F. & Townsend, R. F. (1940). *Lancet*, **i**, 1074.

Common, R. H. (1934). *Nature, Lond.*, **133**, 572.

Common, R. H. (1936). *J. agric. Sci.* **26**, 472.

Common, R. H. (1941). *J. agric. Sci.* **31**, 412.

Couch, J. R. (1955). *Feed Age*, **5**, 55.

Delory, G. E. & King, E. J. (1945). *Biochem. J.* **39**, 245.

Driggers, J. C. & Comar, C. L. (1949). *Poult. Sci.* **28**, 420.

Francis, D. W. (1957). *Poult. Sci.* **36**, 181.

Franseen, C. C., Simmons, C. S. & McLean, R. (1939). *Surg. Gynec. Obstet.* **68**, 1038.

Gutowska, M. S. & Mitchell, C. A. (1945). *Poult. Sci.* **24**, 159.

Hall, G. E. & King, E. J. (1930–31). *Poult. Sci.* **10**, 132.

Hawk, P. B., Oser, B. L. & Summerson, W. H. (1947). *Practical Physiological Chemistry*, 12th ed., p. 584. London: J. and A. Churchill Ltd.

Jowsey, J. R., Berlie, M. R., Spinks, J. W. T. & O'Neil, J. B. (1956). *Poult. Sci.* **38**, 1234.

King, E. J. & Armstrong, R. (1934). *Can. med. Ass. J.* **31**, 376.

McIndoe, W. M. (1957). *Biochem. J.* **67**, 19P.

McIndoe, W. M. (1959). *Biochem. J.* **72**, 153.

Motzok, I. (1950*a*). *Biochem. J.* **47**, 193.

Motzok, I. (1950*b*). *Biochem. J.* **47**, 196.

Motzok, I. (1959). *Biochem. J.* **72**, 169.

Motzok, I. & Wynne, A. M. (1950). *Biochem. J.* **47**, 187.

Ohmori, Y. (1937). *Enzymologia*, **4**, 217.

Peterson, W. J. & Parrish, D. B. (1939*a*). *Poult. Sci.* **18**, 54.

Peterson, W. J. & Parrish, D. B. (1939*b*). *Poult. Sci.* **18**, 59.

Stutts, E. C., Briles, W. E. & Kunkel, H. O. (1957). *Poult. Sci.* **36**, 269.

Wiese, A. C., Johnson, B. C., Elvehjem, C. A., Hart, E. B. & Halpin, J. G. (1939). *J. biol. Chem.* **127**, 441.

Winget, C. M. & Smith, A. H. (1958). *Poult. Sci.* **37**, 509.

Winget, C. M., Smith, A. H. & Hoover, G. N. (1958). *Poult. Sci.* **37**, 1325.

Woodard, H. W. & Higinbotham, N. L. (1941). *J. Amer. med. Ass.* **116**, 1621.

Biochem. J. (1960) **75**, 230

The Effect of 5-Bromouracil Deoxyriboside on the Synthesis of Antibody *in vitro*

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The synthesis of antibody *in vitro*, in common with other protein synthesis, can be inhibited by the addition of amino acid analogues to the medium (Stavitsky & Wolf, 1958; Vaughan, Dutton, Dutton George & Marston, 1960). In an earlier paper it was shown that antibody synthesis could also be inhibited by the purine antagonist 8-azaguanine (Dutton, Dutton & George, 1958). It was established in a spleen-cell system, however, that 8-azaguanine, in addition to inhibiting the synthesis of ribonucleic acid, was affecting other pathways of metabolism and was thus of little use in elucidating biochemical pathways involved in the formation of antibody (Dutton *et al.* 1958).

The present paper describes the inhibition of antibody formation by the thymidine analogue 5-bromouracil deoxyriboside, which may be expected to affect a much more limited number of metabolic pathways. The incorporation of amino acids into the trichloroacetic acid-insoluble fraction of the spleen cells is not affected. The inhibitor appears to affect deoxyribonucleic acid but not ribonucleic acid metabolism. The implications of these findings are discussed.

A preliminary account of part of this work has already been presented (Dutton, Dutton & Vaughan, 1959*a*, *b*).

EXPERIMENTAL

Materials

[^{14}C]*Amino acids.* These were obtained from Nuclear-Chicago Corp., Ill., U.S.A. In most experiments [*carboxy*-^{14}C]DL-leucine was used. In some cases uniformly labelled L-[^{14}C]leucine, L-[^{14}C]lysine or L-[^{14}C]phenylalanine was used. (Specific activities ranged from 0·5 to 5·0 mc/mM.)

Inhibitors. 5-Bromouracil deoxyriboside was obtained from the California Corporation for Biochemical Research, Los Angeles, U.S.A.

Methods

The techniques used for the preparation of the spleen-cell cultures, measurement of antibody synthesis *in vitro* and the uptake of radioactive phosphate are described in detail elsewhere (Vaughan *et al.* 1959; Dutton, Dutton, George, Marston & Vaughan, 1960). The following is a brief description.

Immunization. Rabbits weighing 2–3 kg. were immunized with 16 intravenous injections of alum-precipitated ovalbumin over a period of 4 weeks. One or 2 months later the animals were stimulated with 2 mg. of alum-precipitated ovalbumin.

Spleen-cell preparation and incubation. Two days after the anamnestic stimulation, the animals were killed, the abdomen was opened aseptically and the spleen perfused and removed. A cell suspension was made by mincing the spleen and sieving it through a double 60-mesh screen. The isolated cells were washed three times in phosphate-buffered NaCl soln. containing 2–3% of bovine albumin and were finally resuspended in a modified Eagle's medium containing 15% of normal rabbit serum. Eagle's medium contained penicillin (100 i.u./ml.) and streptomycin (0·1 mg./ml.). Portions of this final suspension were pipetted into 10 ml. beakers. The incubation mixture was made up as follows: cell suspension, containing approx. 3×10^7 cells, 1 ml.; medium, with or without inhibitor, 0·5 ml.; [^{14}C]amino acid, 0·5 μc, dissolved in medium, 0·2 ml. The beakers and contents were incubated at 37° in an atmosphere of $O_2 + CO_2$ (95:5, v/v), saturated with water vapour.

Measurement of antibody synthesis. After incubation for prescribed periods the cells were separated from the supernatant by centrifuging at 150 **g**. The cells were washed once in aq. 0·85% NaCl and frozen until required. The supernatant was recentrifuged at 1000 **g** for 1 hr. at 5°. The radioactive antibody was co-precipitated from the supernatant by the addition of non-radioactive antibody, followed by the amount of antigen required to give maximal precipitation. The washed precipitate was dissolved in 0·25 N-acetic acid, a portion was plated and the radioactivity measured. Repeated determinations of [^{14}C]antibody in the supernatants from duplicate cultures generally agreed well with a standard deviation of one-tenth of the mean. Most determinations have been made in triplicate, the rest in duplicate. The results presented are averages of all replicates, no selection being made against isolated aberrant results.

Measurement of the incorporation of [^{14}C]*amino acid into 'cell protein'.* The washed cells (wet wt. 10–20 mg.) were thawed and were washed three times in 1 ml. of cold 5% trichloroacetic acid, once in hot 5% trichloroacetic acid, twice in acetone, once in ether and were finally dried in an oven at 60°. The fraction thus obtained is referred to below as 'cell protein'. The cell protein was then

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dissolved in 1 ml. of 6 N-HCl (60°, overnight). A portion (0·1 ml.) of this solution was plated on to a 5 cm.² disk for counting.

Measurement of the incorporation of [^{32}P]*phosphate into the cell-phosphate fractions.* Approximately 50 μc of [^{32}P]-phosphate was added to cultures containing 2×10^8 cells, after the desired incubation period. The cells were incubated for an additional 1 hr. period and then harvested. The phosphate fractions of the cells were separated by a modification of the Schmidt and Thannhauser technique (see Dutton *et al.* 1960). The specific activity of the phosphate in these fractions was determined.

Measurement of radioactivity. Samples containing ^{14}C were counted in a D 47 gas-flow counter (Nuclear-Chicago Corp.) used with a Micromil window. The samples were plated so thinly that no correction for absorption was necessary.

Samples containing ^{32}P were counted in a 20th Century Electronics liquid counter, type M6.

RESULTS

It has been shown (Vaughan *et al.* 1960) that spleen cells, isolated as described, will synthesize antibody *in vitro* for at least 48 hr., although in occasional experiments (four out of seventeen) the rate of synthesis falls off after 24 or 36 hr. In the experiments to be described the spleen cells were isolated 2 days after the anamnestic stimulation *in vivo* and incubated *in vitro* for a period of 48 hr. Under these conditions (Fig. 1) the rate of antibody synthesis is slow initially but increases approximately threefold during incubation.

Inhibition of antibody production. When the incubation medium contained 5-bromouracil deoxyriboside the antibody produced in a 48 hr. period was reduced to about one-third of the control value. The effect of concentration of the inhibitor is shown in Fig. 2. It can be seen that as little as 0·02 mM caused marked inhibition. An increase up to 2·5 mM did not reduce antibody

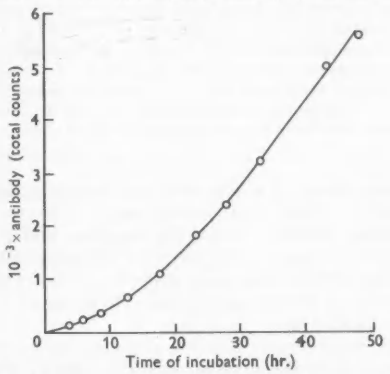

Fig. 1. Incorporation of [^{14}C]amino acid into antibody synthesized by spleen cells incubated *in vitro*. The spleen cells were removed from rabbit 138 two days after anamnestic stimulation *in vivo* and were incubated in medium containing [^{14}C]amino acids. Individual cultures containing the spleen-cell suspension were removed after varying periods of incubation and the radioactivity of the antibody, recovered from the supernatant, was estimated.

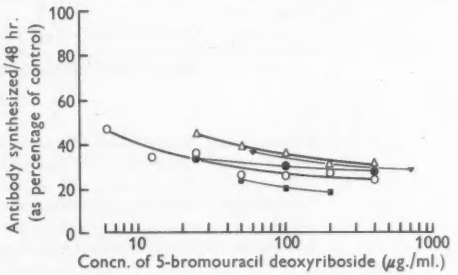

Fig. 2. Effect of the concentration of 5-bromouracil deoxyriboside on the amount of antibody synthesized. Spleen cells isolated from rabbits 2 days after anamnestic stimulation *in vivo* were incubated for 48 hr. with [^{14}C]-amino acids and varying concentrations of 5-bromouracil deoxyriboside. The amount of radioactive antibody in the supernatant at 48 hr. was measured. Results obtained with five different rabbits are illustrated: ○, R 302; ●, R 309; △, R 125; ▼, R 153; ■, R 140.

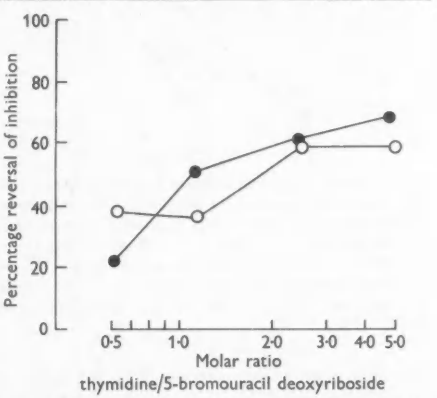

Fig. 3. Reversal of the 5-bromouracil deoxyriboside-inhibition of antibody synthesis by thymidine. The amount of antibody made by spleen cells isolated from rabbit 302 2 days after anamnestic stimulation and incubated for 48 hr. *in vitro* was measured for uninhibited cells (x), for cells inhibited with 5-bromouracil deoxyriboside (y), and for cells in which the inhibition was reversed by the further addition of varying concentrations of thymidine (z). The percentage reversal of inhibition, $(z-y)/(x-y) \times 100$, is plotted against the molar ratio of thymidine/5-bromouracil deoxyriboside. Measurements were made at two concentrations of 5-bromouracil deoxyriboside, 6 μg./ml. (○) and 50 μg./ml. (●).

Table 1. *Effect of uridine and thymidine on the inhibition of antibody synthesis by 5-bromouracil deoxyriboside*

Spleen cells were incubated with [^{14}C]amino acid for 48 hr. under the conditions indicated and the radioactivity incorporated into antibody in this period was determined.

Additions to the medium	Antibody (total counts)
Rabbit 131	
None	980
5-Bromouracil deoxyriboside (350 μg./ml.)	340
5-Bromouracil deoxyriboside (350 μg./ml.) plus uridine (350 μg./ml.)	443
5-Bromouracil deoxyriboside (350 μg./ml.) plus thymidine (350 μg./ml.)	813
Rabbit 148	
None	7235
5-Bromouracil deoxyriboside (200 μg./ml.)	3340
5-Bromouracil deoxyriboside (100 μg./ml.)	2805
5-Bromouracil deoxyriboside (200 μg./ml.) plus uridine (350 μg./ml.)	3115
5-Bromouracil deoxyriboside (100 μg./ml.) plus uridine (175 μg./ml.)	3205
5-Bromouracil deoxyriboside (200 μg./ml.) plus thymidine (350 μg./ml.)	4380
5-Bromouracil deoxyriboside (100 μg./ml.) plus thymidine (175 μg./ml.)	6030

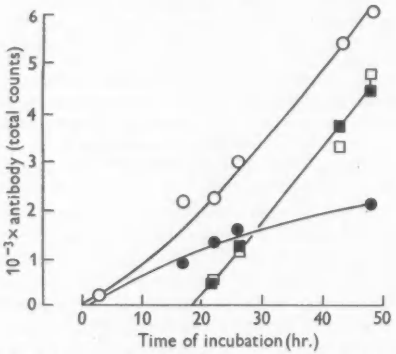

Fig. 4. Effect of time of addition of 5-bromouracil deoxyriboside on the synthesis of antibody. The antibody synthesized by '2-days spleen cells' incubated *in vitro* is plotted against the time of incubation. In ○, ●, [^{14}C]amino acids and inhibitor were added at 0 hr. and in □, ■, [^{14}C]amino acids and inhibitor were added at 17 hr. ○, □, Controls; ●, ■, with 5-bromouracil deoxyriboside (50 μg./ml.). Rabbit 132.

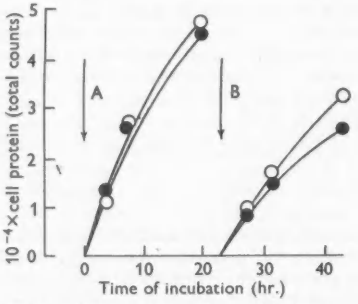

Fig. 5. Effect of 5-bromouracil deoxyriboside on the incorporation of [^{14}C]amino acid into 'cell protein' of spleen cells incubated *in vitro*. ○, Control; ●, incubated with 5-bromouracil deoxyriboside (50 μg./ml.). In series A, [^{14}C]amino acids were added at 90 min.; in series B, at 24 hr. 5-Bromouracil deoxyriboside was added to the inhibited cultures of both series at 0 hr. Rabbit 144.

synthesis appreciably further. The inhibition was partially reversed by the presence of thymidine (Fig. 3), but not by uridine (Table 1). The reversal with thymidine appeared to be competitive, although the system is not sufficiently defined to allow an inhibition analysis.

Effect of time of addition of 5-bromouracil deoxyriboside. In several experiments the time course of inhibition of antibody synthesis by 5-bromouracil deoxyriboside was studied. The result of one such experiment, which was typical, is illustrated in Fig. 4. When the inhibitor and [^{14}C]leucine were added at the beginning of incubation (Fig. 4) the incorporation of amino acid into antibody lagged steadily behind that of the control cultures. In marked contrast, when the inhibitor and [^{14}C]-leucine were added to a portion of this same spleen-cell suspension after incubation for 17 hr. (Fig. 4) there was little demonstrable inhibition of antibody synthesis.

It was thought possible that the cells might release thymidine, or an enzyme capable of destroying the inhibitor, into the medium during the course of incubation. However, if fresh cells were suspended in old medium, i.e. the supernatant obtained from cells after incubation for 24 hr., the initial inhibiting effect observed with 5-bromouracil deoxyriboside was not reduced. Furthermore, the antibody production of cells already incubated for

Table 2. *Effect of 5-bromouracil deoxyriboside on the uptake of [^{32}P]phosphate into phosphate fractions of spleen cells*

Cultures were incubated with or without inhibitor. At 2, 16 or 28 hr. [^{32}P]phosphate was added and the cultures were incubated for a further 60 min. The cells were then harvested, the phosphate fractions isolated and the specific activities determined. The antibody produced in parallel cultures, incubated for 48 hr., was also measured.

Rabbit no.	Incubation time (hr.) before addition of radioactive phosphate		Specific activity reached after incubation for 60 min. with [^{32}P]phosphate (counts/μg. of P)				Radioactive amino acid incorporated into antibody (total counts 48 hr.)
			Acid-soluble phosphate	Fat-soluble phosphate	Ribonucleic acid phosphate	Deoxyribonucleic acid phosphate	
R 140	2	Control	780, 710 } 745	65, 61 } 63	72, 64 } 68	3·6, 3·9 } 3·8	4024
		5-Bromouracil deoxyriboside (200 μg./ml.)	700, 610 } 655	73, 62 } 68	68, 60 } 64	3·9, 3·9 } 3·9	724
		Inhibition (%)	12	−8	6	−3	82
R 135	16	Control	480, 540 } 510	52, 84 } 68	38, 45 } 42	2·7, 3·0 } 2·9	5177
		5-Bromouracil deoxyriboside (200 μg./ml.)	500, 570 } 535	65, 76 } 71	36, 37 } 37	2·0, 2·1 } 2·1	2008
		Inhibition (%)	−5	−4	12	28	60
R 149	28	Control	534, 550 } 542	47, 73 } 60	33, 33 } 33	2·0, 2·9 } 2·5	503
		5-Bromouracil deoxyriboside (200 μg./ml.)	570, 560 } 565	53, 51 } 52	32, 28 } 30	1·4, 1·3 } 1·4	235
		Inhibition (%)	−4	13	9	34	53

24 hr. and resuspended in fresh medium was still not inhibited by 5-bromouracil deoxyriboside added at this time.

Incorporation of amino acid into cell protein. The incorporation of [^{14}C]leucine into the trichloroacetic acid-insoluble residue of the cell is illustrated in Fig. 5. In this experiment the inhibitor was added at the beginning of incubation and [^{14}C]-amino acids were added at either 90 min. (Fig. 5, A) or at 24 hr. (Fig. 5, B). It can be seen that the presence of the inhibitor caused no significant inhibition of cell-protein synthesis even after prolonged incubation.

Incorporation of phosphate into the phosphate fractions of spleen cells. Owing to difficulties of technique, differences in specific activities of less than 15 % cannot be regarded as significant. This is especially true when the measurement is made several hours after incubation has begun. The reductions in the specific activity of deoxyribonucleic acid phosphate at 16 hr. and 28 hr., however, are 28 and 34 % (Table 2). There was no demonstrable effect by the inhibitor on the uptake of [^{32}P]phosphate into acid-soluble, fat-soluble or ribonucleic acid phosphate fractions even after incubation for 28 hr. with 5-bromouracil deoxyriboside at 200 μg./ml. These findings are in contrast with the effect of 8-azaguanine (Dutton *et al.* 1958), which, at 300 μg./ml., had brought about a 30 % reduction of incorporation into acid-soluble phosphate, 28 % reduction of incorporation into ribonucleic acid phosphate and a complete elimination of incorporation into deoxyribonucleic acid after only 4 hr.

DISCUSSION

In the experiments described, the spleen cells were isolated 2 days after anamnestic stimulation with antigen. Under these conditions the rate of antibody formation *in vitro* increased approximately threefold during the first 24 hr. of incubation. In contrast, a constant rate of antibody formation was described when cells were isolated at 3 days (Vaughan *et al.* 1960). The increasing rate of synthesis occurring during incubation of cells obtained at 2 days can be interpreted in two ways. Either the templates utilized in the synthesis of antibody protein are working faster, or more templates are coming into use.

The total amount of antibody produced in incubation for 48 hr. by the '2-day' spleen cells is greatly reduced by the presence of 5-bromouracil deoxyriboside. The degree of inhibition is little affected by the concentration of the inhibitor over the wide range tested. The production of antibody in this system thus appears to be divisible into two parts. The synthesis of approximately one-third is little affected by the presence of the inhibitor, whereas the production of the other two-thirds is strongly inhibited by the inhibitor even in low concentrations (Fig. 2). It is tempting to surmise that the increasing rate of antibody synthesis in the '2-days system' is due to the development of increased number of templates, and that 5-bromouracil deoxyriboside selectively inhibits this development.

The time study of the inhibition is consistent with this possibility. When the inhibitor was added at the beginning of the incubation period the rate of antibody production failed to show the usual increase. Instead, a slight fall was seen. However, if the inhibitor is added after the rate of antibody synthesis has already increased, there is little or no effect on the subsequent synthesis (Fig. 4).

5-Bromouracil deoxyriboside is a structural analogue of thymidine and has been shown to inhibit the utilization of this compound for deoxyribonucleic acid synthesis in bacteria (Bardos, Levin, Herr & Gordon, 1955; Beltz & Visser, 1955, 1957) and in mammals (Kit, Beck, Graham & Gross, 1958; Eidinoff, Knoll & Marano, 1957). It has been shown both in bacteria (Dunn & Smith, 1957; Litman & Pardee, 1956; Price, Hudson, Hinds, Darmstadt & Zamenhof, 1956) and in mammalian tissues (Eidenoff, Cheong & Rich, 1959) that 5-bromouracil deoxyriboside, or 5-bromouracil, is, under certain conditions, incorporated into deoxyribonucleic acid, partially replacing thymidine. The effect of such incorporation on the genotype and phenotype of a cell needs clarification (for a discussion see Zamenhof, De Giovanni & Rich, 1956).

It seems reasonable to assume that in the present experiments 5-bromouracil deoxyriboside inhibits cell division by inhibiting spleen-cell synthesis of deoxyribonucleic acid although there has been, as yet, no direct demonstration of cell division in the spleen-cell system. It is possible that some few cells may synthesize deoxyribonucleic acid containing 5-bromouracil, or may undergo division in the absence of deoxyribonucleic acid synthesis (cf. the findings of Linder, 1959, with 5-fluorouracil). It is likely that either of these last-named metabolic complications, however, would result in abnormal or deficient cytoplasmic function and thereby inhibit antibody formation.

It has generally been considered that thymidine is incorporated solely into deoxyribonucleic acid (Reichard & Estborn, 1951; Friedkin, Tilson & Roberts, 1956). It has also been suggested that thymidine may have some other metabolic function (Prusoff, 1957; Beltz & Visser, 1957). However, Kit *et al.* (1958), working with mouse spleens, rat thymus and tumour cells, were unable to find evidence of such other function. 5-Bromouracil deoxyriboside had no effect on the methylation of

deoxyuridine to thymidine, the hydroxymethylation reaction leading to serine synthesis, the anabolic reactions involved in the incorporation of [^{14}C]formate into protein or the energy-yielding processes of glycolysis and respiration. In their experiments, the cells were incubated for only 3 hr. In the present study, during the 48 hr. no effect could be observed on the uptake of [^{32}P]phosphate into cell fractions other than deoxyribonucleic acid. Also the incorporation of [^{14}C]amino acid into cell protein was not affected by the inhibitor, making it seem unlikely that thymidine or some derivative is involved in activation and incorporation of amino acids into protein.

In summary, there is a stage in antibody formation which is independent of inhibition by 5-bromouracil deoxyriboside. This is indicated by the persistence of significant antibody formation even when the concentration of inhibitor is raised many times above the minimal inhibitory level (Fig. 2), and in the complete lack of inhibitory effect in preparations allowed to incubate through a critical time interval (Fig. 4). It would seem that the inhibitor does not inhibit antibody formation, but affects an earlier developmental stage associated with increasing capacity to form antibody.

The mechanism by which 5-bromouracil deoxyriboside brings about this inhibition is not yet established. The inhibitor may affect some as yet undemonstrated function of thymidine or a thymidine derivative necessary for the development of synthetic ability. The inhibitor does inhibit the synthesis of new deoxyribonucleic acid. This inhibition may prevent the division of cells which either are, or become, capable of antibody formation. Further experiments are needed to differentiate these alternatives.

SUMMARY

1. The rate of synthesis of antibody by rabbit-spleen cells, isolated 2 days after anamnestic antigenic stimulation *in vivo*, increases roughly threefold during the first 24 hr. of incubation *in vitro*.

2. This increase is prevented by 5-bromouracil deoxyriboside and the inhibition appears to be reversed competitively by the addition of thymidine.

3. The incorporation of radioactive amino acid into cell protein, the synthesis of antibody and the incorporation of radioactive phosphate into cell-phosphate fractions other than deoxyribonucleic acid, do not appear to be affected by the inhibitor.

4. The implications of these findings are discussed.

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REFERENCES

Bardos, T. J., Levin, G. M., Herr, R. R. & Gordon, H. L. (1955). *J. Amer. chem. Soc.* **77**, 4279.

Beltz, R. E. & Visser, D. W. (1955). *J. Amer. chem. Soc.* **77**, 736.

Beltz, R. E. & Visser, D. W. (1957). *J. biol. Chem.* **226**, 1035.

Dunn, D. B. & Smith, J. D. (1957). *Biochem. J.* **67**, 494.

Dutton, R. W., Dutton, A. H. & George, M. (1958). *Nature, Lond.*, **182**, 1377.

Dutton, R. W., Dutton, A. H., George, M., Marston, R. Q. & Vaughan, J. H. (1960). *J. Immunol.* (in the Press).

Dutton, A. H., Dutton, R. W. & Vaughan, J. H. (1959*a*). *Fed. Proc.* **18**, 565.

Dutton, R. W., Dutton, A. H. & Vaughan, J. H. (1959*b*). *Fed. Proc.* **18**, 219.

Eidinoff, M. L., Cheong, L. & Rich, M. A. (1959). *Fed. Proc.* **18**, 220.

Eidinoff, M. L., Knoll, J. & Marano, B. J. (1957). *Proc. Amer. Ass. Cancer Res.* **2**, 198.

Friedkin, M., Tilson, D. & Roberts, D. (1956). *J. biol. Chem.* **220**, 627.

Kit, S., Beck, C., Graham, O. L. & Gross, A. (1958). *Cancer Res.* **18**, 598.

Linder, A. (1959). *Cancer Res.* **19**, 189.

Litman, R. M. & Pardee, A. B. (1956). *Nature, Lond.*, **178**, 529.

Price, T. B., Hudson, P. B., Hinds, H. A., Darmstadt, R. A. & Zamenhof, S. (1956). *Nature, Lond.*, **178**, 684.

Prusoff, W. H. (1957). *J. biol. Chem.* **226**, 901.

Reichard, P. & Estborn, B. (1951). *J. biol. Chem.* **188**, 839.

Stavitsky, A. B. & Wolf, B. (1958). *Biochim. biophys. Acta*, **27**, 4.

Vaughan, J. H., Dutton, A. H., Dutton, R. W., George, M. & Marston, R. Q. (1960). *J. Immunol.* (in the Press).

Zamenhof, S., De Giovanni, R. & Rich, K. (1956). *J. Bact.* **71**, 60.

The Proteins of *Hevea brasiliensis* Latex

4. ISOLATION AND CHARACTERIZATION OF CRYSTALLINE HEVEIN*

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Fresh rubber latex obtained from *Hevea brasiliensis* consists of a suspension of several types of particles in an aqueous serum, and can be separated into fractions by high-speed centrifuging (Cook & Sekhar, 1953). The top layer in the centrifuge tube consists mainly of rubber particles, below which is a layer of almost clear serum. At the bottom of the tube is a layer referred to in this paper as the 'bottom fraction', which contains sedimentable bodies including the lutoid particles described by Homans & van Gils (1948).

In preliminary work an aqueous extract of the bottom fraction was found to contain several electrophoretically distinct protein components. This observation has been confirmed and elaborated by Moir & Tata (1960). This paper describes the isolation and characterization of a major component which is anionic at pH 8·6 and for which the name hevein is proposed.

EXPERIMENTAL

Materials. Freeze-dried samples of the bottom fraction of freshly tapped latex were used throughout. Collection of the latex and centrifugal separation and freeze-drying of the bottom fraction were carried out by the staff of the Rubber Research Institute of Malaya, as described by McMullen (1959). All freeze-dried fractions were sealed under high vacuum in Malaya, and on arrival at our Laboratory were stored at $-15°$ until required.

Elementary analyses. Total N was determined by the micro-Kjeldahl method and C, H and ash by Pregl micro-combustion. The S content of the ash and gaseous combustion products was measured turbidimetrically as $BaSO_4$ after complete dissolution of the ash in dilute HCl. Where the amount of material available for analysis was limited, and the ash content was known to be low (as in purified hevein), C, H and S were determined on the same sample with a microcombustion tube containing a roll of silver and PbO_2 only. P was determined colorimetrically with ammonium molybdate after wet oxidation.

Soluble proteins were extracted from the freeze-dried solids with 0·05 M-phosphate buffer, pH 6·8, or with 0·05 M-$Na_2B_4O_7$, pH 9·3. The protein-N content of the extract was then determined by tannic acid precipitation and Kjeldahl analysis (Archer & Sekhar, 1955). Purified protein samples were dried to constant weight *in vacuo* at 80° before analysis.

* Part 3: Moir & Tata (1960).

Paper electrophoresis. For determining the number of electrophoretically distinct proteins in a solution, the method of Kunkel & Tiselius (1951) as modified by Archer & Sekhar (1955) was used. The isoelectric point of hevein was also determined with a paper-strip-electrophoresis technique. This method was chosen because sufficient purified material was not available for the classical Tiselius method in free solution. The buffers used for determining the isoelectric point contained 0·02 M-sodium acetate, together with sufficient acetic acid to give the required values over the range pH 4·38–5·39. All pH values were determined at 20° with a Cambridge pH meter and glass electrode. Current was applied to the standard paper-electrophoresis apparatus by means of platinum electrodes immersed in beakers of electrolyte connected via agar bridges to the buffer compartments of the apparatus. This modification was necessary to prevent pH changes in the buffer solution reaching the paper strips during the long periods of electrophoresis (about 48 hr.), needed because of the low mobility of the protein near the isoelectric point. The voltage across the strip was measured with platinized electrodes immersed in the buffer close to the strip, and connected to a recording voltmeter, thereby enabling the average voltage during an experiment to be determined. Approximately 700 v was applied across the current electrodes, resulting in a potential gradient in the paper itself of 8·5 v/cm. The electrophoretic movement of the protein in a given time was taken as the difference between the distances moved by the protein and a dextran spot which was applied to a second strip placed alongside that containing the protein. All determinations were carried out in duplicate. The pH of zero mobility was obtained by graphical interpolation of the results obtained at five values in the range pH 4·38–5·38.

Isolation of hevein. Approx. 40 g. of freeze-dried bottom fraction was mixed with 360 ml. of O_2-free water, 0·2 g. of sodium dithionite (to deactivate the polyphenol-oxidase enzymes, which rapidly turn the solutions black), 0·05 ml. of octan-1-ol (to reduce frothing) and a few small pieces of solid CO_2 in a high-speed blender (MSE Ato-Mix) at 0° for 1 min. The mixture was then centrifuged below 5° and the residue re-extracted twice more with 80 ml. of cold water as described above. A.R. $(NH_4)_2SO_4$ was then added to the combined extracts in the proportions of 43 g./100 ml. of liquid, with constant stirring. The pH was adjusted to 6·0 and the mixture stored at 0° for 16 hr. The precipitate was rejected after centrifuging and washing it with 65% saturated $(NH_4)_2SO_4$ solution, and the hevein was precipitated from the combined supernatants by saturating with $(NH_4)_2SO_4$. This precipitate was washed with saturated $(NH_4)_2SO_4$ solution and mixed with water until dissolution was almost complete. An equal volume of 65% saturated

$(NH_4)_2SO_4$ was then added and, after standing for 2 hr., any insoluble material was centrifuged off and rejected. The supernatant was again saturated with $(NH_4)_2SO_4$, the precipitated hevein redissolved in 65% saturated $(NH_4)_2SO_4$ and reprecipitated as before. The final precipitate was then dissolved in water and desalted. This could not be achieved satisfactorily by our usual dialysis technique because of leakage of protein through the cellulose membranes, so electrodialysis was employed with membranes whose permeability to hevein was negligible.

Electrodialysis. An apparatus similar to that of Wood (1956) was constructed from Perspex. The 5 cm. diameter electrodes were of carbon and the anode and cathode membranes were Permaplex ion-exchange membranes (The Permutit Co. Ltd., London) A. 10 and C. 10 respectively. Soft water previously cooled to 0° was circulated past the electrodes at a combined rate of about 18 l./hr. The initial protein concentration was restricted to that equivalent to $E^{280\,m\mu}_{1\,mm.}$ 0·3 to reduce membrane blockage, which occurred at higher concentrations. The d.c. voltage applied to the cell from a full-wave rectifier unit (unsmoothed) was initially 60 v and rose to 150 v as the salt was removed. The initial current was 300 mA, which decreased to 7 mA at the end of the electrodialysis.

Crystallization. After electrodialysis, the protein solution was freeze-dried, and the resulting solid dissolved in the minimum volume of water at 40°. The solution was then centrifuged and allowed to crystallize at 0° overnight. The hevein was centrifuged off and recrystallized twice more. The crystals were finally dried *in vacuo* over P_2O_5.

Paper chromatography. The amino acids present in hevein hydrolysates were identified by two-dimensional paper chromatography on Whatman no. 1 paper with phenol–$NaHCO_3$, followed by lutidine–propan-2-ol–diethylamine–water as solvents (see below).

For quantitative determination of the amino acids, a simple one-dimensional descending technique with a variety of solvents was used, similar to that of Roland & Gross (1954) as modified by Drake, Giffee, Johnson & Koenig (1957). Approx. 10 mg. of crystalline hevein (dried to constant weight at 70° *in vacuo*) was accurately weighed and hydrolysed in a sealed tube *in vacuo* with 1 ml. of 90% formic acid and 1 ml. of 17% HCl for 24 hr. at 100°. The hydrolysate was then transferred to a small distillation apparatus and evaporated to dryness under reduced pressure in a stream of N_2. The residue was dissolved in a few drops of water and transferred to a small round-bottomed flask of calibrated volume (0·591 ml.), sealed to the end of a B. 14 standard ground-glass socket. This enabled a volume of up to 2 ml. of water to be used for washing the distillation flask, excess of water in the calibrated flask being removed in a stream of N_2. Finally 0·1 ml. of propan-2-ol was added and the solution diluted with water to the standard volume. This microtechnique was necessitated by the small amounts of hevein available. A micropipette delivering 0·005 ml. was used to put the spots of hydrolysate on the paper and the same pipette was used for the spots of standard amino acids prepared in 10% propan-2-ol solution by direct weighing. The standard amino acid solutions were 20 mM except for tyrosine and glutamic acid, which were 10 mM. Dilutions were prepared at 16 mM, 10 mM, 6 mM and 4 mM. Four sheets of Whatman no. 1 paper, 36 cm. × 54 cm., carried a total of nine spots of hydrolysate and nine spots of each of the standards. After solvent development the sheets were air-dried for 24 hr. and then immersed in 0·25% of ninhydrin in acetone containing 1% of acetic acid. For the determination of proline 0·2% of isatin in acetone was used instead of ninhydrin. The sheets were left in a dark box at 50% relative humidity at room temperature overnight, and the maximum densities of the spots were determined with an EEL scanner converted for use with a 3 mm. diameter aperture instead of the standard slit. Amino acid concentrations were determined (probable accuracy ±5–10%) from curves of maximum scanner-galvanometer readings against standard amino acid concentrations. The solvents used for development were:

A. Phenol–$NaHCO_3$ (Drake *et al.* 1957) in an atmosphere of coal gas, with solutions of NH_3 (3%) and KCN (10%) in beakers in the tanks. Aspartic acid, glutamic acid, serine, glycine, threonine, alanine, tyrosine, histidine and arginine were determined by this method.

B. Butan-1-ol–acetic acid–water (25:6:25, by vol.). The upper phase was used for development and the lower phase for equilibration in the tank. Proline and lysine were estimated with this system.

C. *iso*Amyl alcohol–pyridine–water–diethylamine (100:100:70:4, by vol.) with beakers containing 1% KCN soln. and an aqueous solution of phenol in the tank. This method was used for leucine and to prove the absence of isoleucine.

D. Butan-2-ol–3% NH_3(aq.) soln. (3:1, v/v) was used for determination of valine.

Determination of thiol groups. The amperometric-titration method originally developed by Kolthoff & Harris (1946), as modified by Benesch & Benesch (1948) and Weiseman, Schoenbach & Armistead (1950), was used.

Spectrographic analysis. The spectrum of hevein was studied in neutral, acid and alkaline media in the range from 215 to 350 mμ with a Hilger Uvispek spectrophotometer. The tyrosine and tryptophan contents of the unhydrolysed protein were calculated from the spectral curves in acid and alkaline media as described by Beaven & Holiday (1952), Goodwin & Morton (1946) and Bencze & Schmid (1957).

Ultracentrifuge analysis. Sedimentation measurements were carried out at 21° on a 0·7% solution of hevein in 0·2 M-NaCl at pH 6·0 in a Spinco model E ultracentrifuge, with an artificial boundary cell, at 59 780 rev./min.

Osmometry. The apparatus used was similar to that of Adair (Alexander & Johnson, 1949). To prevent diffusion of hevein through the nitrocellulose membranes the latter were treated with an 18% glycerol solution in water (Adair, 1955) to reduce their specific permeability. The most suitable value for the latter was found to be $1–4 \times 10^6$ c.g.s. units. Equilibrium osmotic pressures were determined at 0° in 0·2 M-NaCl at pH 5·0 over a range of concentrations with recrystallized hevein. The concentration of the solution inside the membrane was determined by u.v. absorption after the osmotic pressure had been measured.

RESULTS

The paper-electrophoresis pattern (Fig. 1) and the sedimentation diagram (Fig. 2) of crystalline hevein give no indication that more than one protein component is present. The elementary

analysis of hevein (Found: C, 46·0; H, 6·0; N, 15·7; S, 5·0; $P < 0·006$; ash, 0·2%) is notable for the high sulphur content.

Reactions of the protein to the Molisch, Benedict and Fehling tests were all negative, indicating the absence of carbohydrate.

Amino acid analysis. The 15 amino acids detected in hevein hydrolysate by paper chromatography are listed in Table 1. No evidence was found for the presence of isoleucine, norleucine, norvaline, asparagine, ornithine, phenylalanine, cysteine, methionine, dihydroxyphenylalanine or hydroxyproline, and they are assumed to be absent.

The thiol-titration method was shown to give consistent results with '*tert.*-dodecylmercaptan' on samples containing as little as 50 μg. of S. With bovine-serum albumin the method gave 0·65 mole of SH/mole of albumin (uncorrected for water content of the protein) as compared with a value of approx. 0·67 obtained by Hughes (1947). When 6 mg. of hevein, containing 330 μg. of total S, was titrated a zero end-point was obtained. A similar titration with standard mercuric chloride solution instead of silver nitrate gave the same result. This confirms the absence of cysteine, and since methionine is also absent the whole of the S in hevein has been attributed to cystine.

All the figures in Table 1 were obtained from the paper-chromatographic maximum-density results with the exception of cystine, tryptophan and tyrosine. The last two were calculated from the spectral curves (see below).

Ultraviolet-absorption spectra. Fig. 3 shows the spectra of hevein in 0·05 N-sodium hydroxide and in 0·05 N-hydrochloric acid. The curve in pure water is indistinguishable from that in the acid medium. The absence of an inflexion at 257·5 mμ in aqueous or acid solutions corroborates the chromatographic evidence that phenylanine is absent ($E^{1\%}_{1\,cm.}$ 25·6 in aqueous solution for the main peak at 280 mμ). The percentages of tryptophan in the hevein molecule, calculated from the spectra by the three methods referred to in the Experimental section, were 9·6, 9·0 and 8·6 respectively; the corresponding values for tyrosine were 4·0, 4·3 and 4·0.

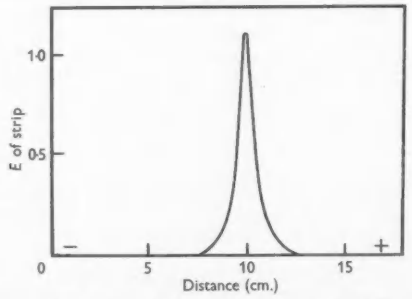

Fig. 1. Paper electrophoresis of hevein. Electrophoresis was carried out for 16 hr. at pH 8·6 in 0·05 M-veronal buffer.

Table 1. *Amino acid analysis of hevein*

	Percentage (w/w) of residues	Relative proportions of amino acid residues
Alanine	1·1	1·6
Arginine	5·7	3·7
Aspartic acid	11·6	10·1
Cystine	19·2	8·0
Glutamic acid	9·9	7·8
Glycine	4·4	7·8
Histidine	3·8	2·8
Leucine	4·7	4·2
Lysine	5·4	4·2
Proline	4·1	4·2
Serine	8·7	10·0
Threonine	1·8	1·8
Tryptophan	9·1	4·6
Tyrosine	4·1	3·8
Valine	1·3	1·3
	94·9	

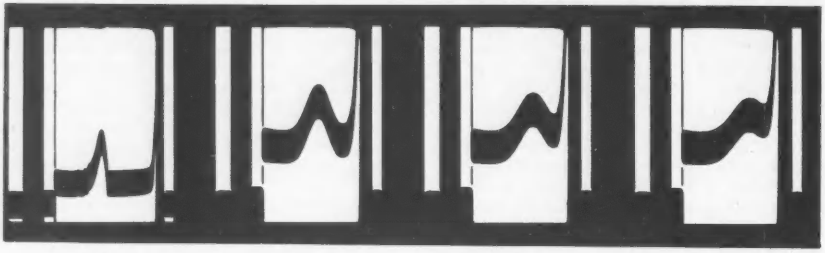

Fig. 2. Ultracentrifugal analysis of hevein at 59 780 rev./min. with a 0·7% solution of the protein in 0·2 N-NaCl at pH 6·0. S $1·19 \times 10^{-13}$.

Osmotic pressure. Equilibrium was reached in the osmometer in about 12 days, and by extrapolating a plot of π/c against c to zero concentration, a value of the apparent molecular weight in 0·2 M-sodium chloride at pH 5·0 of 10 500 was obtained.

Isoelectric point. The pH at which hevein had the same mobility as dextran in 0·02 M-sodium acetate buffer, in paper-electrophoresis experiments, was 4·7.

Solubility. The solubility of pure hevein in water at 0° is less than 1 %, but the solubility increases rapidly in the presence of neutral salts. The protein crystallizes readily from salt-free cold water in the form of hexagonal needles (Fig. 4), which show birefringence. It is appreciably soluble in ammonium sulphate solutions which are below saturation and its solutions are not coagulated by heat, although its solubility in strong ammonium sulphate solutions is lowered by this treatment.

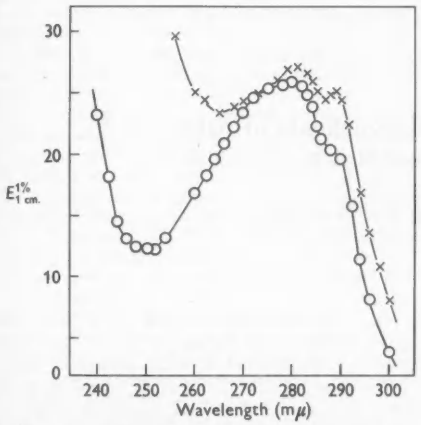

Fig. 3. Ultraviolet-absorption spectra of hevein: ○, in 0·05 N-HCl; ×, in 0·05 N-NaOH.

Fig. 4. Photomicrograph of hevein crystals.

DISCUSSION

Assuming that the nearest integers to the numbers in the third column of Table 1 represent the numbers of amino acid residues in the hevein molecule, a minimum molecular weight of 9512 is obtained, which may be compared with the values of 10 500 calculated from the osmotic data.

In respect of its high sulphur content and low molecular weight, hevein resembles the trypsin-inhibiting protein from lima beans reported by Fraenkel-Conrat, Bean, Ducay & Olcott (1952).

The electrophoretic and solubility properties of hevein suggest that this protein may be identical with a protein component (no. 4) identified by Archer & Sekhar (1955) in the aqueous serum of *Hevea brasiliensis* latex, and work is in progress to elucidate this point. It also appears that hevein is largely responsible for the band designated component (viii) by Moir & Tata (1960).

SUMMARY

1. A crystalline protein has been isolated from fresh rubber latex (*Hevea brasiliensis*), for which the name hevein is proposed.

2. This protein contains 5 % of sulphur, has a molecular weight of approximately 10 000, a sedimentation constant of 1·19 S at a concentration of 0·7 %, and $E^{1\%}_{1\,cm.}$ at 280 mμ 25·6.

3. Hevein contains 15 amino acids including cystine but no cysteine or methionine.

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REFERENCES

Adair, G. S. (1955). *Biochem. J.* **62**, 26.

Alexander, A. E. & Johnson, P. (1949). *Colloid Science*, vol. 1, p. 164. Oxford: Clarendon Press.

Archer, B. L. & Sekhar, B. C. (1955). *Biochem. J.* **61**, 503.

Beaven, G. H. & Holiday, E. R. (1952). *Advanc. Protein Chem.* **7**, 369.
Bencze, W. L. & Schmid, K. (1957). *Analyt. Chem.* **29**, 1193.
Benesch, R. & Benesch, R. E. (1948). *Arch. Biochem.* **19**, 35.
Cook, A. S. & Sekhar, B. C. (1953). *J. Rubb. Res. Inst. Malaya*, **14**, 163.
Drake, M. P., Giffee, J. W., Johnson, D. A. & Koenig, V. L. (1957). *J. Amer. chem. Soc.* **79**, 1395.
Fraenkel-Conrat, H., Bean, R. C., Ducay, E. D. & Olcott, H. S. (1952). *Arch. Biochem. Biophys.* **37**, 393.
Goodwin, T. W. & Morton, R. A. (1946). *Biochem. J.* **40**, 628.
Homans, L. N. S. & van Gils, G. E. (1948). *Proc. 2nd Rubb. Tech. Conf.* p. 292.
Hughes, W. L. jun. (1947). *J. Amer. chem. Soc.* **69**, 1836.
Kolthoff, I. M. & Harris, W. E. (1946). *Industr. Engng Chem. (Anal.)*, **18**, 161.
Kunkel, H. G. & Tiselius, A. (1951). *J. gen. Physiol.* **35**, 89.
McMullen, A. I. (1959). *Biochem. J.* **72**, 545.
Moir, G. F. J. & Tata, S. J. (1960). *J. Rubb. Res. Inst. Malaya* (in the Press).
Roland, J. F. & Gross, A. M. (1954). *Analyt. Chem.* **26**, 502.
Weiseman, N., Schoenbach, E. B. & Armistead, E. B. (1950). *J. biol. Chem.* **187**, 153.
Wood, T. (1956). *Biochem. J.* **62**, 611.

Biochem. J. (1960) **75**, 240

Methods for Measuring Rates of Synthesis of Albumin by the Isolated Perfused Rat Liver

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Although the ability of the isolated perfused rat liver to synthesize plasma proteins was clearly demonstrated in 1951 by Miller, Bly, Watson & Bale, few such experiments have subsequently been reported. The potential use of this system for studies on protein synthesis was, however, recognized by Jensen & Tarver (1956). These authors added ^{14}C-labelled histidine and lysine to the blood used for perfusing a liver, and then measured the specific activity of the free amino acids in the plasma at regular intervals. With these data, and by measuring the corresponding amounts of the [^{14}C]amino acid which had become protein-bound, they were able to calculate a rate of protein synthesis.

However, their measurements of the specific activities of the free amino acids in the plasma, which were obtained by means of the bacterial decarboxylase method, may have been subject to considerable experimental error. For this reason it seemed worth while to repeat some of their measurements with the aid of a more accurate estimation for the specific activity of the free amino acids in the plasma.

The results thus obtained could be compared with the rate of albumin synthesis *in vivo* found by Campbell, Cuthbertson, Matthews & McFarlane (1956). Since it seemed possible that the perfused liver may on average synthesize albumin either faster or slower than it does *in vivo*, it seemed desirable also to measure albumin synthesis directly in the perfusion system. To achieve this, isolation of the newly formed albumin was necessary. Newly formed rat albumin cannot be separated from the rat albumin initially present in the system when the perfusion is conducted with rat blood. Thus the expedient of perfusion with a heterologous blood became necessary. Human blood from which most of the white cells and platelets had been removed proved satisfactory for liver perfusions and permitted the isolation of newly formed rat albumin by means of an appropriate antiserum. Although such a system suffers from certain disadvantages mentioned below, its use has permitted direct comparison between rates of synthesis of albumin calculated from changes in plasma-amino acid specific activity and from the amount of albumin obtained by isolation in the same experiment.

METHODS

Perfusion technique. Perfusions were carried out as described by Cohen & Gordon (1958), except for the use of human blood in certain cases. Carrier lysine was used in all experiments except rat-liver perfusion (RLP) 87 (for amounts see Table 1). When human blood was used the white cell and platelet layer, which separated from the heparinized blood during preliminary centrifuging at 1800 *g* for 20 min. at 20°, was removed as completely as possible by suction and the plasma was re-mixed with the red cells. Warm Ringer's solution was allowed to flow through the liver for 1–2 min. before its removal from the

donor rat, in order to remove as much as possible of the rat blood initially present.

Injection of liver-donor rat with rat [^{131}I]*albumin.* In order to estimate the quantity of rat albumin remaining in the liver, despite the preliminary perfusion with Ringer's solution, rat [^{131}I]albumin (approx. 5 mg. containing 150 μc of ^{131}I) was injected into the donor rat on the day before the perfusion experiment. It was assumed that by the time the liver was removed all extracellular albumin present would have become mixed with ^{131}I-labelled albumin to the same extent as that in the serum, thereby enabling it to be recognized and measured. The principle involved here was used by Humphrey & Sulitzeanu (1958) for isolation of intracellular antibody from the tissues of hyperimmunized rabbits. Labelling with ^{131}I was carried out by the ICl method of McFarlane (1958) at a level of not more than 1 atom of iodine/molecule of albumin.

Isolation and estimation of rat albumin. This was carried out by precipitation with an antiserum, prepared by injection of purified rat albumin into rabbits, and containing about 3 mg. of anti-rat albumin/ml. Since the original antiserum precipitated some of the α-globulins as well as the albumin it was pretreated by addition of rat α-globulin (60 mg./100 ml.). This material was kindly prepared by Dr S. Cohen by chromatography on carboxymethylcellulose prepared according to Peterson & Sober (1956). A portion (5 ml.) of the pretreated antiserum was added to a sample (2 ml.) of the human plasma obtained at the end of the perfusion. Sodium ethylenediaminetetra-acetate (0·4 ml. of 1% solution), to prevent coagulation, carrier lysine (10–20 mg.) and heparin (0·05 ml., 250 i.u.), were also present. After 2 hr. at 37° the precipitate was removed on the centrifuge and washed three times at 2° with 0·9% NaCl. It was decomposed, and the albumin was recovered in solution, by addition of 2 ml. of 1% trichloroacetic acid in aq. 96% (v/v) ethanol (Delaville, Delaville & Delaville, 1954; Schwert, 1957). The albumin-containing solution was dialysed against water for 48 hr. in Visking dialysis tubing which had previously been soaked and rinsed in water to remove preservatives. The recovered rat albumin was freeze-dried and redissolved in 1 ml. of water. From this solution samples were taken for estimation of protein by the method of Lowry, Rosebrough, Farr & Randall (1951) and measurement of ^{131}I-radioactivity and of ^{14}C-radioactivity after combustion to CO_2 (Bradley, Holloway & McFarlane, 1954). The efficiency of this method for the recovery of pure rat albumin was tested by adding ^{131}I-labelled rat albumin of known specific radioactivity to human plasma, recovering the albumin in the way described and comparing the specific radioactivity of the recovered material with that of the original. Since rat α-globulin was the most probable contaminant unlabelled α-globulin was also added to the mixture. The procedure was as follows: 2·37 mg. of rat [^{131}I]albumin + 2 mg. of rat α-globulin were added to 2 ml. of human plasma. This was mixed with 15 ml. of rabbit antiserum against rat albumin, containing heparin (0·1 mg./ml.) and left overnight at 2°. The amount of antibody present was sufficient to precipitate 5 mg. of rat albumin, but the antiserum also contained some antibodies against other rat-plasma proteins. After washing the antigen–antibody precipitate twice on the centrifuge with 15 ml. of cold 0·9% NaCl 96% of the radioactivity was recovered in the complex. The washed precipitate was stirred with 6 ml. of 1% (w/v) trichloroacetic acid in 96% (v/v) ethanol at 2° and the undissolved protein separated after 10 hr. by centrifuging; the precipitate was extracted with a further 2 ml. of trichloroacetic acid–ethanol mixture, and the clear supernatants were combined. To this solution, which contained 35% of the radioactivity of the original antigen–antibody precipitate, was added 2 vol. of ether at 2°, and albumin precipitate, which formed in a few minutes, was separated by centrifuging. It was dissolved in 1 ml. of water containing a drop of M-$NaHCO_3$, and samples were taken for estimation of protein radioactivity. The specific radioactivity of the recovered rat albumin was 86% of that of the starting material, measured at the same time. This indicates that the rat albumin recovered by this technique was largely, but not quite, free from contaminating protein. The conditions of the experiment were designed to be rather more exacting than in the actual liver-perfusion experiments, since the ratio of rat α-globulin to albumin was several times higher than that in normal plasma, and the technique was judged sufficiently specific to warrant its use in the work described below.

Estimation of [^{14}C]*lysine specific activity.* Samples of plasma of 2–10 ml. (depending on the expected lysine concentration) were stirred with 2 vol. of 10% trichloroacetic acid and centrifuged. The whole of each supernatant, less 0·5 ml. which was kept for amino acid estimation by the ninhydrin method (Jacobs, 1956), was allowed to run through a 0·3 cm. × 7·0 cm. column of Zeo-Karb 225 (4½% cross-linked, passing 200 mesh). The resin was pretreated with hot NaOH and HCl according to Moore, Spackman & Stein (1958), and was used in the acid form after extensive washing with water. After removal of all the trichloroacetic acid from the column by passage of water the amino acids were eluted with 5 ml. of aq. 3 N-NH_3 soln. Complete removal of the NH_3 was next carried out by repeated evaporations on the steam bath. Finally the solid material was dissolved in approx. 0·05 ml. of water and transferred, as completely as possible, to a 3 cm. wide strip of Whatman no. 3 MM filter paper arranged for paper electrophoresis. Because such paper may contain large quantities of material reacting with ninhydrin, the strips were prewashed in several litres of dilute Na_2CO_3 solution, water and finally 0·01 M-Na_2CO_3; 0·01 M-Na_2CO_3 was also used as the buffer for the subsequent paper electrophoresis. The strips were not dried before use for the paper electrophoresis [which was carried out for 21 hr. at 500 v in the apparatus described by Flynn & de Mayo (1951)]. Next the strips were dried at a temperature not exceeding 100° and scanned for radioactivity. Adequacy of separation of lysine and arginine was checked by means of the ninhydrin colour which was developed on control strips. Possibly because very dilute Na_2CO_3 was used, the absolute positions of the amino acids varied considerably, but the relative positions of lysine and arginine remained reasonably constant. It was therefore possible to cut off a part of each strip containing only lysine. Care was necessary, however, to obtain cuts containing only lysine, since not all of the radioactivity appeared in the lysine band (see below). Elution of the lysine from the selected part of each strip yielded solutions which were divided for ^{14}C-counting and amino acid estimation. Because volumes of less than 0·1 ml. were sufficient for accurate estimation of radioactivity, and because it was necessary to add carrier carbon for this purpose, the solutions were allowed to soak into small pieces of filter paper placed ready in the platinum boats.

Care was taken during the ^{14}C estimations to avoid loss of $^{14}CO_2$, so that the count obtained, appropriately corrected for transfer losses, represented the whole of the radioactivity in the sample. The values for total radioactivity thus obtained, divided by the amounts of lysine present, gave the specific activity of the free lysine in each plasma sample. The accuracy of this method for measurement of lysine specific activity was tested by isolation of [^{14}C]lysine from a synthetic mixture of amino acids including arginine, sufficient lysine to give amounts of approx. 50 μg. of amino acid for each ninhydrin estimation being employed. The mean specific activity of the isolated [^{14}C]lysine thus found was $95 \pm 4{\cdot}8$ % of the original sample.

Extraction of intracellular lysine. Immediately after perfusion the livers were chopped coarsely with scissors and rapidly frozen in liquid air. After transfer to a cooled mortar and addition of 25 ml. of 20 % trichloroacetic acid the liver was ground thoroughly as soon as the rising temperature permitted. Contamination with extracellular lysine was neglected in view of the much higher concentration of all amino acids within the liver cells than in lymph or plasma.

Estimation of radioactivity in proteins. ^{131}I-Radioactivity was measured in a standard volume, in a well-type scintillation counter.

^{14}C-Radioactivity was measured after combustion to CO_2 (Bradley *et al.* 1954), thereby permitting ^{14}C to be measured in samples containing ^{131}I. To ensure removal of non-protein ^{14}C in plasma samples they were first treated as described by Miller *et al.* (1951).

Calculations. The rate of synthesis of albumin has been calculated in three different ways. In method *A* the calculation is based on the specific activity of the ^{14}C-labelled rat albumin isolated from the plasma at the end of each experiment and the total ^{14}C-radioactivity of this plasma. Since the proportion of radioactivity present as albumin was also measured in RLP 90 and in a subsequent experiment (see below) the total ^{14}C present as albumin in the final plasma could be calculated. Division of the total albumin radioactivity by the albumin specific activity (Table 2) gives the amount of newly synthesized albumin together with the amount transferred from the liver donor. This latter quantity can be estimated, since the donor albumin was labelled with ^{131}I, and can be subtracted to give a value for the amount of albumin newly synthesized during the perfusion.

In method *B* (Table 3) the calculation is based on the amount of rat albumin isolated by means of the antiserum, estimated by the method of Lowry *et al.* (1951). Losses occurring during the isolation are measured and corrected by estimation of ^{131}I-radioactivity at each stage, since rat albumin was the only ^{131}I-labelled protein present. As in method *A*, it is necessary to subtract the amount of albumin transferred from the liver donor.

In method *C* (Table 4) the rate of synthesis of albumin has been derived from data for the increases in the ^{14}C-radioactivity of the plasma albumin during each hour of the experiment, together with the average specific activities of the free lysine in the plasma during each corresponding time interval. From these can be calculated the weight of lysine incorporated into plasma proteins during each hour, on the assumption that the plasma-lysine specific activity was the same as the specific activity of the lysine at the site of synthesis. How nearly this assumption is valid is considered below.

RESULTS

Ratio of [^{14}C]lysine incorporated into albumin and total plasma protein by the perfused liver. A portion (3 ml.) of the final plasma (human blood) from RLP 90, in which [^{14}C]lysine was present, was subjected to electrophoresis on a column of treated cellulose (Porath, 1954). When the protein was eluted the peak of human albumin (detected by absorption at 280 mμ) was, as expected, somewhat ahead of the peak of rat albumin, which was estimated by scintillation-counting. The fractions corresponding to the u.v.-absorption peaks were pooled and concentrated. Their ^{14}C-radioactivities were measured and protein content was estimated by the biuret method (Gornall, Bardawill & David, 1949). The proportion of the radioactivity appearing in the leading fraction was 60 %, which was shown by means of electrophoresis on paper to

Table 1. *Details of individual perfusions*

Blood donors had not been starved before use. [^{14}C]Lysine (uniformly labelled; 25 μc) was added at the start of each perfusion. Bile was collected from the cannulated bile duct in all perfusions except nos. 87 and 92.

			Perfusion blood						
						Concn. of free amino acid (mg./100 ml. of plasma)			
Blood used	Perfusion no.	Liver donor body wt. (g.)	Vol. before sampling (ml.)	Initial haemato-crit value (%)	Initial concn. of plasma protein (g./100 ml.)	Initial	Final	Time perfused after addition of [^{14}C]lysine (hr.)	L-Lysine added (mg.)
Rat	93	290	74	38	7·0	73	138	4·33	7·5
	97	345	75	37	7·3	62	132	4·33	6·8
	1	295	64	40	7·4	—	—	4·33	6·2
	8*	315	72	41	7·2	68	134	4·42	5·6
Human	87	315	50	39	8·8	58	176	3·66	0
	88	360	61	44	9·0	54	177	4·0	6·8
	90	300	72	43	7·9	88	210	4·25	6·8
	92	280	74	44	8·0	80	172	4·25	6·5

* Penicillin and streptomycin sulphate (2·5 mg. each) were added initially.

Table 2. *Calculations of rate of synthesis of albumin from amounts isolated after perfusion with human blood*

Method *A*: not depending on recovery of ^{131}I

Perfusion no.	87	88	90	92
Plasma in circuit minus samples (ml.)	26·6	28·1	26·6	27·5
Final plasma-protein ^{14}C (μc/ml.)	$8{\cdot}41 \times 10^{-3}$	$5{\cdot}87 \times 10^{-3}$	$6{\cdot}50 \times 10^{-3}$	$4{\cdot}73 \times 10^{-3}$
Total protein ^{14}C in circuit, including samples (μc)*	$2{\cdot}41 \times 10^{-1}$	$1{\cdot}84 \times 10^{-1}$	$2{\cdot}12 \times 10^{-1}$	$1{\cdot}57 \times 10^{-1}$
(1) Albumin ^{14}C-radioactivity at 64% of total protein radioactivity (μc)†	$1{\cdot}54 \times 10^{-1}$	$1{\cdot}18 \times 10^{-1}$	$1{\cdot}36 \times 10^{-1}$	$1{\cdot}00 \times 10^{-1}$
(2) ^{14}C specific activity of isolated rat albumin (μc/g.)	3·18	1·96	2·04	2·18
Albumin synthesized (uncorrected), i.e. (1)/(2) (mg.)	48·4	60·0	66·2	46·1
Albumin transferred from liver donor (mg.)‡	11·0	11·6	10·5	10·8
Therefore, by subtraction, albumin synthesized/4 hr. (mg.)	37·4	48·4	55·7	35·3
Albumin synthesized/hr. (mg.)	9·3	12·1	13·9	8·8
Average rate of albumin synthesis (mg./hr.)		11·0		

* No correction has been made for free [^{14}C]lysine removed in samples, on the grounds that it represents a relatively small fraction of the total in the system, including the liver itself.

† See Results section.

‡ Calculated from ^{131}I in donor-rat albumin.

Table 3. *Calculation of rate of synthesis of albumin from amounts isolated*

Method *B*

Perfusion no.	87	88	90	92
Rat albumin in circuit recovered by specific precipitation with antibody and estimated by method of Lowry, Rosebrough, Farr & Randall, 1951 (uncorrected) (mg.)	32·0	44·8	31·4	25·8
Correction factor from ^{131}I recovery	0·552*	0·469	0·575	0·616
Rat albumin in circuit corrected by ^{131}I recovery (mg.)	58·0	95·6	64·6	41·8
Albumin transferred from liver donor (mg.)	11·0*	11·6	10·5	10·8
By subtraction, albumin synthesized/4 hr. (mg.)	47·0	84·0	44·1	31·0
Albumin synthesized/hr. (mg.)	11·7	21·0	11·0	7·8
Average rate of albumin synthesis (mg./hr.)		12·9		

* No ^{131}I label was used in this experiment. The correction factor used is the average of that in the other three experiments.

Table 4. *Example of method of calculating rate of synthesis of albumin from plasma-lysine ^{14}C specific activities and incorporation of lysine ^{14}C into total plasma proteins at hourly intervals (RLP 92; human blood)*

Method *C*

Time (hr.)	1	2	3	4
Protein C in perfusion circuit (mg.) (*a*)	1400	1297	1178	1075
μc of ^{14}C/mg. of C entering all plasma proteins (*b*)	$7{\cdot}10 \times 10^{-5}$	$6{\cdot}55 \times 10^{-5}$	$4{\cdot}82 \times 10^{-5}$	$2{\cdot}73 \times 10^{-5}$
Mean plasma lysine C specific activity during each hour (μc/mg. of C) (*c*)	0·531	0·312	0·159	0·108
Lysine C incorporated (mg.) into all plasma proteins (*ab*)/*c*	0·19	0·28	0·36	0·28
Albumin (mg.) synthesized* $\left(\frac{ab}{c} \times \frac{10 \times 0{\cdot}64 \times 100}{\text{C (\%) in albumin}}\right)$	2·42	3·58	4·62	3·58
Rate of albumin synthesis				
Average over 4 hr. (mg./hr.)		3·54		

* Assuming (*a*) that lysine C composes 10% of all plasma-protein C (this figure is calculated from corresponding figures for human-plasma proteins, taken from Spector, 1956); (*b*) that albumin ^{14}C composes 64% of all plasma-protein ^{14}C (experimental result); (*c*) that the rate of labelling of albumin and of the total plasma proteins is the same.

consist only of albumin. The remaining 40% of the radioactivity was in later fractions containing mostly α- and β-globulin, but also a trace of albumin. In order to allow for the radioactivity of the albumin which had trailed, the percentage of total radioactivity incorporated into albumin was taken as 64%.

Comparison of livers perfused with human and with rat blood. The main information is contained in Tables 1–4. It is evident that the rat liver when perfused with human blood still retains its ability to synthesize albumin and at least some of the plasma globulins. However, those livers which had been perfused for 4 hr. with human blood could

Table 5. *Comparison of rates of synthesis of albumin*

Blood used	Perfusion no.	Average plasma free-amino acid concn. (mg./100 ml.)	Percentage of dose incorporated into all plasma proteins in 4 hr.	Albumin synthesized (mg./hr./300 g. rat): Isolation of newly formed albumin (method *A*)	Albumin synthesized (mg./hr./300 g. rat): Plasma lysine specific activity (method *C*)
Rat	93	105	10·1	—	—
	97	97	12·0 (With samples)	—	—
	1	—	16·1	—	4·8 } 5·0
	8	101	15·2	—	5·2 } (average)
Human	87	135	16·1*	9·3	—
	88	133	11·9 (With samples)	12·1	—
	90	149	15·0	13·9	6·4 } 4·9
	92	126	11·3	8·8	3·5 } (average)
Rat	Jensen & Tarver (1956)	—	—	—	13·1†

* No extra lysine was added to perfusion.

† Assuming that 64% of the plasma-protein radioactivity at the end of the perfusion is in the form of albumin, and that the mean lysine content of rat-plasma proteins is 10%.

usually not be freed of blood so completely by a final perfusion with 0·9% NaCl as could livers which had been perfused similarly with rat blood. Furthermore, although the average initial total free amino acid level of the human blood used in these experiments was close to that of the rat blood, this level rose considerably higher at the end of the experiments with human blood (cf. Table 1). Despite these differences certain comparisons between the two systems are worth making. The average percentage of the added [^{14}C]lysine which became incorporated into plasma protein was almost the same in the perfusions with rat (13·3%) or human (13·6%) bloods. Comparison of the specific activities of the free lysine of plasmas obtained during perfusion with human and with rat blood reveals no differences attributable to the bloods used. Furthermore, the average rate of albumin synthesis calculated (method *C*) from the specific radioactivities of the free lysine of plasma are very similar for the livers perfused with the two kinds of blood (Table 5). In fact the difference calculated in this same way between RLP 90 and 92, both of which were perfused with human blood, was much greater than the difference between the average for human and the average for rat blood. With only a pair of experiments of each kind this may be accidental, but the ratios between the rates of albumin synthesis in experiments RLP 90 and 92, calculated by methods *A* and *C*, and the ratio of the percentages of the dose of [^{14}C]lysine incorporated into total plasma protein in the same two perfusions, are rather similar.

Rates of synthesis of albumin. The amounts of rat albumin isolated by means of the antiserum from the perfusions with human blood have been calculated in Tables 2 and 3 by methods *A* and *B* given above. Assuming that the albumin isolated was pure (the impurities amounted almost certainly to less than 10%; see under Methods) the accuracy of these calculations should depend only on the accuracy of the methods for estimation of (1) ^{14}C-radioactivity (Bradley *et al.* 1954), (2) protein concentration of the isolated albumin (Lowry *et al.* 1951), (3) ^{131}I-radioactivity, together with (4) the estimate of the proportion of ^{14}C occurring as albumin in the final plasma.

The calculated amounts of albumin synthesized in perfusions 87, 88, 90 and 92 calculated by methods *A* and *B* are more variable by method *B* than *A*. It seems likely that the figures obtained by method *A*, which are based on direct measurements of total ^{14}C-activity in the perfusing blood, are the more accurate. In any case the average rates of albumin synthesis calculated by the two methods are in substantial agreement (cf. Tables 2 and 3).

The specific activities of the free lysine of plasma measured at various times during four of the liver perfusions are shown in Fig. 1. These values have been used to calculate the rate at which lysine is incorporated into plasma albumin, and so to estimate the rate of albumin synthesis, with the results shown in Table 5. In perfusions 90 and 92 the average rate thus calculated is approximately two-fifths of that obtained by the method of isolating the albumin. As mentioned above, calculation by method *C* takes no account of any dilution of the [^{14}C]lysine with the unlabelled lysine which is continuously produced from preformed proteins inside the liver cell. The true specific activity of intracellular lysine is difficult to measure with certainty, both because the liver must first be freed of blood, which process may itself

alter the intracellular-amino acid specific activity, and also because any abnormality of environment or partial damage to the liver may accelerate catabolism of all kinds of intracellular proteins. As shown by Fig. 1, the specific activity of free lysine in plasma varies considerably from one perfusion to another. Measurements of intracellular-lysine specific activity were at least as variable; an average of six experiments gave a value 55% (range 17–73%) of the corresponding average for the specific activity of free lysine in plasma. If the rates of albumin synthesis calculated by method *C* are corrected to allow for intracellular-lysine specific activities half those found in the plasma, values more nearly in agreement with, but still lower than, those obtained by isolation are obtained.

Appearance of lysine-degradation product during perfusion. Rather surprisingly the paper-electrophoresis method used for the separation of lysine from the other amino acids present in the plasma demonstrated the presence among the bases of a radioactive substance not corresponding with either lysine or arginine. The amount of this substance in the plasma increased during the course of each perfusion. Under the conditions for electrophoresis used its mobility was sufficiently greater than that of lysine to permit nearly complete separation. In most perfusions, even after 4 hr., the amount present did not exceed 20% of the total radioactivity of the isolated lysine. Attempts to detect such a component in the liver itself were unsuccessful. Some further data on the properties of this substance, which suggest that it may be an amine, will be presented in a separate paper. Whether it is formed by the liver itself or by the anaerobic bacteria usually found in this organ in the rat is not known. The latter possibility seems unlikely, since the material was formed in perfusion 8, to which both penicillin and streptomycin had been added.

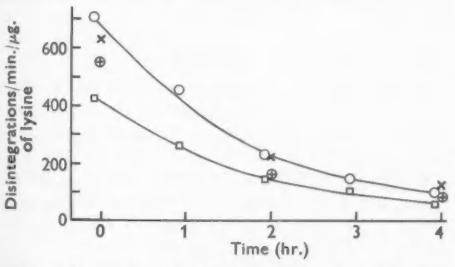

Fig. 1. ^{14}C specific activity of plasma lysine at various times after addition to perfusion blood. ○, □, Human blood; ×, ⊕, rat blood. $2{\cdot}2 \times 10^6$ disintegrations/min. $\equiv 1\,\mu c$.

DISCUSSION

The first aim of the present investigation was to attempt to confirm the results of Jensen & Tarver (1956) on the rate of synthesis of plasma protein by the perfused liver.

On the basis of experiments in which [^{14}C]lysine was used these authors reported a rate of synthesis of 31·4 mg. of total plasma protein/hr. by the liver of a 322 g. rat. In the present work the average rate of synthesis of total plasma protein by the livers of two only slightly smaller rats (RLP 1 and 8), which were perfused with rat blood, was found to be 7·8 mg./hr. The method of calculation used to obtain this figure is the same as that given for albumin in Table 4, but omitting the correction for the proportion of albumin to total protein synthesized. Recalculation of Jensen & Tarver's data on lysine incorporation with the assumptions and by the method shown in Table 4 (method *C*) gives 20·6 mg. of total plasma protein/hr., which is nearly three times the value calculated by this method from our results. In view of the considerable discrepancy between these two sets of results it is of special interest to compare each with the rate of plasma-protein synthesis *in vivo* reported for the strain of rats in question. Thus Jensen & Tarver (1956) gave the rate of synthesis of the plasma proteins observed in the perfused liver as only slightly below (95%) the maximum rate observed *in vivo* in an experiment in which ^{14}C-labelled plasma was given to rats maintained on diets containing three different levels of protein. The rate of synthesis is taken as the biological half-life of plasma protein after transfer. However, as shown by Freeman & Matthews (1958), this involves certain rather unlikely assumptions about the rate of loss of albumin from the intra- and extra-vascular pools. Dr S. Cohen (unpublished observations) has compared the rate of catabolism of albumin in rats calculated in this way with the results of calculations by the equilibrium–time method (Campbell *et al.* 1956). The latter method was found to give a rate of only 63% of that derived from the apparent biological half-life. Thus it seems reasonable to conclude that the actual maximum rate of plasma-protein synthesis of the strain of rats used by Jensen & Tarver may have been nearer 20 mg. than the suggested 33 mg./hr. In addition it would appear from the data of Steinbock & Tarver (1954) that the rate of protein catabolism is greatly increased in rats on a 65% protein diet compared with that in rats on a normal diet. Although these findings have not been confirmed by D. P. Cuthbertson & A. S. McFarlane (unpublished work), such a difference would imply that had Jensen & Tarver's rats been maintained on a diet similar to that of our rats their protein

turnover would have been substantially lower than the rate which they quote. These considerations greatly reduce the difference between the synthesis rates *in vivo* of the two strains of rats under comparison but make Jensen & Tarver's (1956) observations of a rate of synthesis of plasma protein of 31·4 mg./hr. by the perfused liver unaccountably high. For the strain of rats used in the present work more accurate figures are available for the rate of synthesis *in vivo* of albumin than for the synthesis of the plasma proteins taken together (Campbell *et al.* 1956). As shown in Table 5, RLP 1 and 8, in which rat blood was used, gave rates of albumin synthesis of 4·8 and 5·2 mg./hr. when calculated from the plasma-lysine specific activities, i.e. only an average of 55 % of the rate of synthesis *in vivo* of this protein (8·9 mg./hr.). That our results are nearer to the true rate than those of Jensen & Tarver (1956) is, however, suggested by the findings with those livers which were perfused with human blood. Thus the amounts of albumin isolated from four such experiments represent an average rate of synthesis of 11 mg./hr. or 128 % of the rate observed *in vivo*. If it is therefore assumed that in these perfusions synthesis is taking place at least as fast as *in vivo* it is interesting that the rate of albumin synthesis calculated from the plasma-lysine specific activities is again very low. In fact the average for perfusions 90 and 92 is once again 55 % of the rate of albumin synthesis *in vivo*. Since, however, tissue intracellular-amino acid specific activities *in vivo* are always found to be below the corresponding values for plasma [cf. Loftfield & Eigner (1958) for rat liver and Askonas & Humphrey (1958) for rabbit lung] and similar results now obtained for lysine in the perfused rat liver, the synthesis rates should probably be corrected for this factor. It is in any case difficult to be sure that the specific activities of intracellular amino acids obtained from a whole tissue reflect the specific activities of the amino acids at the sites of protein synthesis, and thus an additional element of uncertainty is inevitable in calculations made by this method.

On the basis of our mean figure (intracellular free-lysine specific activity 55 % of that in plasma after 4 hr. perfusion), the correction would be about twofold. Our results would then fall more nearly into line with those obtained by isolation of the albumin. On the other hand the rate of synthesis of the total plasma proteins given by Jensen & Tarver (1956) when treated in the same way becomes 44 mg./hr. for a rat of 322 g. It should be noted that in order to make the above comparison with the results of Jensen & Tarver (1956) it has been necessary to multiply the rate of protein synthesis of the perfused liver given by them by a factor of 0·7, to compensate for their assumption that rat plasma contained 7 % of lysine instead of 10 % of lysine as assumed in the present work.

As shown in Table 5 the average plasma free-amino acid concentrations in the perfusions with human blood were somewhat higher than when rat blood was used. This fact may account for the ability of the livers under these conditions to synthesize albumin somewhat faster than has been observed *in vivo* for the same strain of rats. On the other hand, neither the specific activity of the plasma-free lysine nor the rate of albumin synthesis calculated on this basis (method *C*) differed much between the experiments with rat and with human blood. Thus livers perfused in these two ways apparently behave similarly, which appears to justify the use of heterologous blood for experiments aimed at studying variation of rates of synthesis by isolation of newly synthesized albumin.

The present work indicates that part of the lysine remaining free in the plasma is progressively transformed under the conditions of our experiments into some other substance, which is presumably unavailable for protein synthesis. This fact will need to be taken into account in any final comparisons between rate of albumin synthesis as measured by incorporation of lysine by the perfused liver and *in vivo*, but does not seem to constitute a likely explanation for the discrepancy between our results and those of Jensen & Tarver (1956). Evidently the accumulation of a radioactive degradation product from [^{14}C]lysine in the blood during liver perfusion is a reason against the use of this amino acid in such work. Thus repetition of the present work with another precursor such as [^{14}C]histidine as was used by Jensen & Tarver (1956) would seem to be worth while.

Our work, together with that of Jensen & Tarver, indicates that the rate of protein synthesis by the isolated perfused liver is sufficiently near to that in the living animal to warrant the use of such a system for further study of the factors governing protein synthesis *in vivo*.

SUMMARY

1. The rate of synthesis of albumin by the isolated perfused rat liver has been calculated from estimates of the free [^{14}C]lysine specific activities in the plasma.

2. Livers have been perfused both with rat blood and with human blood. Newly synthesized albumin has been isolated from the latter experiments by means of an antiserum.

3. Liver-donor rats were previously injected with ^{131}I-labelled rat albumin to allow measurement of the amount of rat albumin transferred to the perfusion circuit with the liver.

4. In perfusions with human blood the average rate of synthesis of albumin, measured by direct isolation, was 12·9 mg./hr. for a liver from a 300 g. rat. By an indirect method of calculation, based on the specific radioactivity of isolated albumin and the total radioactivity incorporated into plasma protein, it was 11 mg./hr. In perfusions with rat or human blood the average rate of synthesis of albumin, calculated from the amounts of [^{14}C]-lysine incorporated into plasma protein and the specific activities of free lysine in the plasma, was 5 mg./hr. The possibility is discussed that the difference is due to the fact that the specific activity of lysine at the site of synthesis was less than that in the plasma.

5. A non-protein ^{14}C-labelled basic substance other than lysine has been found to accumulate during these perfusions.

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REFERENCES

Askonas, B. A. & Humphrey, J. H. (1958). *Biochem. J.* **70**, 212.
Bradley, J. E. S., Holloway, R. C. & McFarlane, A. S. (1954). *Biochem. J.* **57**, 192.
Campbell, R. M., Cuthbertson, D. P., Matthews, C. M. & McFarlane, A. S. (1956). *Int. J. appl. Radiat. Isotopes*, **1**, 66.
Cohen, S. & Gordon, A. H. (1958). *Biochem. J.* **70**, 544.
Delaville, M., Delaville, G. & Delaville, J. (1954). *Ann. pharm. franç.* **12**, 109.
Flynn, F. V. & de Mayo, P. (1951). *Lancet*, ii, 235.
Freeman, T. & Matthews, C. M. E. (1958). *Strahlentherapie*, **38**, 283.
Gornall, A. C., Bardawill, C. J. & David, M. M. (1949). *J. biol. Chem.* **177**, 751.
Humphrey, J. H. & Sulitzeanu, B. D. (1958). *Biochem. J.* **68**, 146.
Jacobs, S. (1956). *Analyst*, **81**, 502.
Jensen, D. & Tarver, H. (1956). *J. gen. Physiol.* **39**, 567.
Loftfield, R. B. & Eigner, E. A. (1958). *J. biol. Chem.* **231**, 925.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
McFarlane, A. S. (1958). *Nature, Lond.*, **182**, 53.
Miller, L. L., Bly, C. G., Watson, M. L. & Bale, W. F. (1951). *J. exp. Med.* **94**, 431.
Moore, S., Spackman, D. H. & Stein, W. H. (1958). *Analyt. Chem.* **30**, 1185.
Peterson, E. A. & Sober, H. A. (1956). *J. Amer. chem. Soc.* **78**, 751.
Porath, J. (1954). *Acta chem. scand.* **8**, 1813.
Schwert, G. W. (1957). *J. Amer. chem. Soc.* **79**, 139.
Spector, W. S. (1956). *Handbook of Biological Data*. London: W. B. Saunders Co.
Steinbock, H. L. & Tarver, H. (1954). *J. biol. Chem.* **209**, 127.

Biochem. J. (1960) **75**, 247

The Extraction of Pigments from *Euglena gracilis*

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Euglena gracilis is a flagellated green alga commonly employed in the microbiological assay of vitamin B_{12}. As a preliminary to assay, biological fluids are ideally diluted to contain about 10 $\mu\mu$g. of vitamin B_{12}/ml. and a series of standards covering a range of 0·25–50 $\mu\mu$g./ml. is in general use. Growth is usually assessed in a colorimeter 6–7 days after commencement of the assay (Ross, 1952; Hutner, Bach & Ross, 1956). The response thus measured is dependent both on turbidity and colour of the *Euglena* suspension.

In the course of experiments on potential vitamin B_{12} antimetabolites (Timmis & Epstein, 1959; Epstein & Petrow, 1960), a series of compounds was screened for inhibitory activity against *E. gracilis* grown at low concentrations of vitamin B_{12}. Compounds found active on screening were subsequently tested again to determine whether their antagonism was competitive in nature and therefore susceptible to reversal by massive vitamin concentrations. In this type of experiment, however, both growth and pigment production, particularly in the standards, became so prolific that the ensuing response could no longer be assessed by the methods referred to above (i.e. measuring both turbidity and colour). The object of this paper is to present a simple and sensitive pigment-extraction technique by which this difficulty may be obviated.

EXPERIMENTAL

Assay of vitamin B_{12}. The test organism was *Euglena gracilis* var. *z*. The method, including basal growth medium, was based on that described by Hutner *et al.* (1956).

Standard vitamin B_{12} solutions covering a range of 0–1000 $\mu\mu$g./ml. were tested in triplicate. The solutions (4 ml., consisting of 2 ml. of double-strength basal medium and 2 ml. of double-strength vitamin standard in water) were incubated in capped Pyrex tubes for 6 days at 28°, after which 4 ml. of acetone was added to each tube. The tubes were then sealed with the thumb and mixed by vigorous shaking for approx. 5 sec. After 10 min. 3 ml. of ethyl acetate was added and the tubes were again shaken for a further 5 sec. After 10 min. the tubes were quickly shaken again and the green supernatant solvent layer was transferred to a cuvette with a 5 mm. light path and read in an EEL (Evans Electroselenium Ltd.) colorimeter at 660–680 mμ (filter no. 608).

Some estimates of growth at low concentrations of vitamin B_{12} were made by the method of Hutner *et al.* (1956) (i.e. measuring both turbidity and pigment), with the same colorimeter and filter; this method is referred to throughout the paper as the standard method. The two methods were also compared at high concentrations of vitamin B_{12} by diluting the cultures. Serial dilutions of cultures grown with 100 and 1000 $\mu\mu$g. of vitamin/ml. were read and extinctions plotted against concentration.

All results reported in this paper were obtained in triplicate and only mean readings are given.

Examination of pigments. The absorption curve of pigment extracted as described above was measured in a Hilger Uvispek spectrophotometer.

Chromatographic analyses of pigment extracts were carried out on sucrose-impregnated Whatman no. 1 filter paper (Sporer, Freed & Sancier, 1954), in the dark at room temperature. The chromatograms were examined both in visible and ultraviolet light.

Measurement of cell numbers and cell size. Algae were counted in a Fuchs–Rosenthal chamber after being killed by the addition of one drop of diluted (1:400) 40% formaldehyde soln. to each tube.

By a modified Biggs & MacMillan (1948) technique, cell-size distributions of 100 algae from cultures grown respectively with 0, 10, 100 and 1000 $\mu\mu$g. of vitamin B_{12}/ml. were measured. Cell images were projected orthogonally on to 22 in. × 30 in. sheets of 1 in. graph paper by a Leitz micro-projector. The projected cells were outlined in pencil and cut out and weighed. The mean weight of six separate 1 in. × 1 in. squares from different parts of each sheet of paper was used for calibration in conjunction with a projected micron scale. One sheet of paper only was used for each concentration of vitamin B_{12}. In this way it was possible to express the recorded weights in terms of square microns (surface area).

Application of the extraction technique to antimetabolite studies. This was studied with from 0 to 500 μg. of 6-mercaptopurine/ml., at 100 μg. intervals, each concentration of 6-mercaptopurine being tested in the presence of 0, 10, 100 and 1000 $\mu\mu$g. of vitamin B_{12}/ml. respectively. The growth response at 6 days was assessed by the extraction method.

RESULTS

Pigments extracted by solvent. Pigment is completely extracted from algal cultures by the method described above, even in the tubes containing 1000 $\mu\mu$g. of vitamin B_{12}/ml.; there was no colour in the cellular residues and aqueous phase. Separation into two layers is rapid and complete. The disrupted cells sediment to the bottom of the assay tubes, and the solvent layer at the interface is debris-free. The green supernatant layer (approx. 3·5 ml.) is translucent. The bands of the absorption spectrum of the extract resembled those observed in solutions of chlorophylls *a* and *b* in other solvents (Zscheile & Comar, 1941; Sprecher von Bernegg, Heierle & Almasy, 1935).

The extinctions of the coloured extract at serial dilutions were determined in the colorimeter and also at two peak absorption bands (670 and 420 mμ) in the spectrophotometer. A linear relationship was obtained at 670 mμ and in the colorimeter up to extinctions of 2·0 and 0·7 respectively, and the colour is stable at room temperature for 12 hr. At 420 mμ, however, where linearity is equally good, absorption is too great to allow estimation at the required concentrations (Fig. 1).

The chromatograms of freshly extracted pigments and of pigments stored at room temperature in the light for 12 hr. are identical.

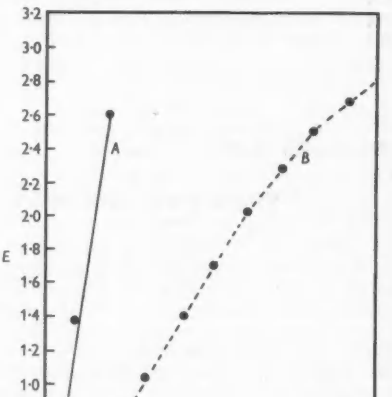

Fig. 1. Relationship between concentrations of extracted pigment from *Euglena gracilis* and extinction. The extract was prepared from a culture of *Euglena* containing 1000 $\mu\mu$g. of vitamin B_{12}/ml. The extract was diluted with acetone. *A*, *B*, Readings at 420 and 670 mμ in the spectrophotometer; *C*, colorimeter reading.

Table 1. *Comparison of results of standard and extraction methods for measuring growth response of* Euglena gracilis

Readings were measured in an EEL colorimeter, filter no. 608. Each result is the mean of 18 estimations.

Concn. of vitamin B_{12} ($\mu\mu$g./ml.)	Standard method: Colorimeter readings	Standard method: Coefficient of variation (%)	Extraction method: Colorimeter readings	Extraction method: Coefficient of variation (%)
5	24·5	7·0	12·5	6·5
10	47·0	4·6	27·0	5·0

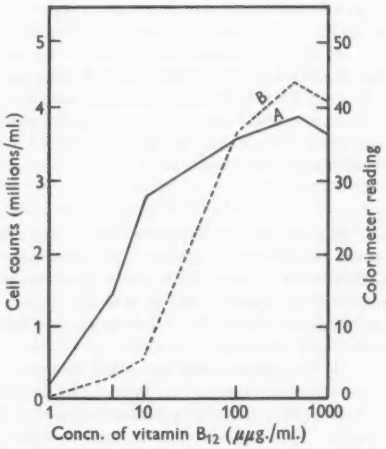

Fig. 2. Comparison between colorimeter readings of extracted pigments from cultures of *Euglena gracilis* (*A*) and cell counts from these same cultures (*B*).

Relation between extracted pigment and vitamin B_{12} concentration. Colorimeter readings of extracted pigments from algal cultures were compared with cell counts from the same cultures. Pigment production does not appear to increase as much as cell population between 10 and 100 $\mu\mu$g. of vitamin B_{12}/ml. (Fig. 2).

Standard and extraction methods were performed in the same assay tubes at low concentrations of vitamin B_{12}; both methods gave comparable results (Table 1). Comparisons at high concentrations of vitamin B_{12} could not be effected; in the standard method of assay not only was excessive dilution required to obtain readings in the colorimeter, but also such dilutions did not show a linear relationship with concentration.

Variation of cell size with vitamin B_{12} concentration. The mean cell size increases significantly between 0 and 10 $\mu\mu$g. of vitamin B_{12}/ml. and is markedly decreased at 100 and 1000 $\mu\mu$g. of vitamin B_{12}/ml. (Table 2). The standard error of distribution of cell size is large in the absence of the vitamin and small with 100 $\mu\mu$g. of vitamin B_{12}/ml. (Fig. 3).

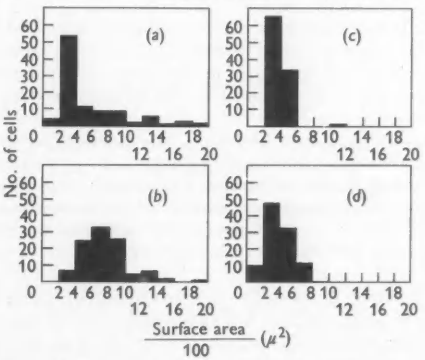

Fig. 3. Distribution of cell size (μ^2) of *Euglena gracilis* at (*a*) 0, (*b*) 10, (*c*) 100 and (*d*) 1000 $\mu\mu$g. of vitamin B_{12}/ml. Each step represents the number of cells at a given surface area and each plot represents the average of results obtained from ten different cultures.

Table 2. *Mean surface area of cells of* Euglena gracilis *at various concentrations of vitamin B_{12}*

The growth period in all cases was 6 days.

Concn. of vitamin B_{12} ($\mu\mu$g./ml.)	Mean surface area (μ^2)	S.D. of distribution
0	455	356
10	687	253
100	299	5·6
1000	326	129

Table 3. *Results of an antagonist experiment with 6-mercaptopurine and the pigment-extraction technique*

Results are expressed as colorimeter readings (EEL). Each value is the mean for three extracts.

Concn. of 6-mercaptopurine (μg./ml.)	Vitamin B_{12} ($\mu\mu$g./ml.): 0	10	100	1000
	Colorimeter readings			
0	2	33	68	69
100	1	28	72	65
200	1	18	68	63
300	0	18	67	60
400	0	10	61	59
500	0	8	60	57

Experiment with 6-mercaptopurine. The results of the antagonist experiment with 6-mercaptopurine are presented in Table 3. It can be deduced graphically from these results that the approximate concentrations of 6-mercaptopurine required to produce 50 % inhibition of growth at 10, 100 and 1000 $\mu\mu$g. of vitamin B_{12}/ml. are 200, >500 and >500 μg./ml. respectively.

DISCUSSION

The method of pigment extraction described in this paper is simple and accurate and can be conveniently applied over a very wide range of concentrations of vitamin B_{12}. The entire procedure is performed in the original assay tube and the extracted pigments are stable. The standard method of assessing the growth response of *E. gracilis* and the extraction method are both equally suitable at the usual range of vitamin B_{12} concentrations assayed in serum, but at high concentrations the standard method is unsatisfactory.

Ostrowki, Skarzynski & Zak (1954) described a methanol method for the extraction of pigment from algal cultures, but this entails centrifuging of individual tubes, and with profuse growth the use of massive volumes of methanol. Similarly, Wolken & Palade (1953) used 80 % acetone or methanol to extract portions of algal cultures. Quantitative assessment of growth at high concentrations of vitamin B_{12} is difficult by the standard method, because of the ensuing high turbidity of *E. gracilis* cultures. Indeed the problem of high turbidities was encountered even at relatively low vitamin concentrations by Hutner *et al.* (1956).

Comparison between pigment concentration and cell counts reveals that these are not directly related. However, it does appear that pigment concentration is related to cell volume; at 10 $\mu\mu$g. of vitamin B_{12}/ml. there is an abundance of pigment per cell with comparatively few cells of large mean diameter but with considerable individual variation in size. At 100 $\mu\mu$g./ml., however, there is relatively little pigment per cell and numerous but small cells of approximately equal sizes. In the standard method of assessing growth response of *E. gracilis*, pigment is measured in conjunction with the turbidity due to the cells present. These cells vary considerably in size with different concentrations of vitamin B_{12} and this fact must contribute an inaccuracy; turbidimetric methods in general depend on the assumption of homogeneity of particle size.

The application of the *E. gracilis* test system to the study of vitamin B_{12} antimetabolites has so far received little attention. This is possibly a reflexion of the technical difficulties in the measurement of growth at high vitamin concentrations. Hendlin & Soars (1951) tested the effect of 1:2-diamino-4:5-dimethylbenzene, 5:6-dimethylbenzimidazole and related compounds on the growth of *Lactobacillus lactis* Dorner and *E. gracilis*. They stated that growth response of *E. gracilis* had been determined turbidimetrically, but no results were given. Funk & Nathan (1958) relied on turbidimetric measurements to observe the inhibition of *E. gracilis* by benzimidazole at high concentrations of vitamin B_{12}, and maintained that under their conditions the growth response was linear at 7 days (H. B. Funk & H. A. Nathan, personal communication).

The antagonist experiment with 6-mercaptopurine illustrates the applicability of the pigment-extraction method to antimetabolite studies. The results indicate that the antagonism is competitive in nature, as it can be partially reversed by high concentrations of vitamin B_{12}.

SUMMARY

1. Pigments were extracted from cultures of *Euglena gracilis* by means of an acetone–ethyl acetate-solvent system. This technique permits the measurement of growth response of this alga over a wider range of vitamin B_{12} concentrations than has hitherto been possible.

2. A direct relationship has been demonstrated between pigment concentration and cell size.

3. The application of the solvent-system technique to vitamin B_{12}-antimetabolite studies is illustrated by an experiment with 6-mercaptopurine.

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REFERENCES

Biggs, R. & MacMillan, R. L. (1948). *J. clin. Path.* **1**, 269.
Epstein, S. S. & Petrow, V. (1960). *Nature, Lond.*, (in the Press).
Funk, H. B. & Nathan, H. A. (1958). *Proc. Soc. exp. Biol., N.Y.*, **99**, 394.
Hendlin, D. & Soars, M. H. (1951). *J. Bact.* **62**, 633.
Hutner, S. H., Bach, M. K. & Ross, G. I. M. (1956). *J. Protozool.* **3**, 101.
Ostrowski, W., Skarzynski, B. & Zak, Z. (1954). *Acta biochim. polon.* **1**, 13.
Ross, G. I. M. (1952). *J. clin. Path.* **5**, 250.
Sporer, A. H., Freed, S. & Sancier, K. M. (1954). *Science*, **119**, 68.
Sprecher von Bernegg, A., Heierle, E. & Almasy, F. (1935). *Biochem. Z.* **283**, 45.
Timmis, G. M. & Epstein, S. S. (1959). *Nature, Lond.*, **184**, 1383.
Wolken, J. J. & Palade, G. F. (1953). *Ann. N.Y. Acad. Sci.* **56**, 873.
Zscheile, F. P. & Comar, C. L. (1941). *Bot. Gaz.* **102**, 463.

Crystalline Lysoplasmalogen (Lysophosphatidal Choline): Preparation from Heart Muscle and Action on Erythrocytes and Spermatozoa

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When mammalian spermatozoa are washed free from seminal plasma (and thus from fructose) and incubated as suspensions in Ringer solution they remain motile provided that oxygen is present. An explanation of this behaviour was put forward by Lardy & Phillips (1941*a*, *b*), who suggested that in the absence of the seminal sugar [later identified by Mann (1946) as fructose] the spermatozoa of bull and ram can derive energy for motility from the oxidative metabolism of intracellular phospholipids.

A study of the lipids present in freeze-dried spermatozoa provided no evidence for the presence of lecithin but showed that the phospholipid fraction consisted mostly of a choline-based plasmalogen (Lovern, Olley, Hartree & Mann, 1957). Subsequently investigations were made into the changes in lipid composition which occur when ram spermatozoa are freed from plasma and incubated as suspensions in Ringer solution (Hartree & Mann, 1958, 1959). The main change observed was a marked decrease in fatty acyl-ester bonds, and from this it was inferred that the reaction responsible for the decrease was the hydrolysis of the sperm plasmalogen to give lysoplasmalogen together with free fatty acid, which, through its oxidative metabolism, could provide the energy necessary to maintain motility.

Lysolecithin has already been shown to have a marked inhibitory action upon the respiration of ram spermatozoa (Dawson, Mann & White, 1957). Since choline lysoplasmalogen can be regarded as a possible product of intracellular-lipid metabolism of ram spermatozoa it was decided to prepare this compound and to compare its properties with those of lysolecithin. An ethanolamine lysoplasmalogen was obtained in crystalline form by Feulgen & Bersin (1939), who believed that they had isolated an unmodified muscle plasmalogen, and again by Rapport, Lerner, Alonzo & Franzl (1957), who established the relationship of the crystalline compound to the native ethanolamine plasmalogen of muscle. We have now obtained from ox-heart muscle the corresponding choline lysoplasmalogen in crystalline form and have studied its action upon erythrocytes and upon ram spermatozoa.

EXPERIMENTAL

Materials

Ram semen was collected and the spermatozoa were separated and washed as previously described (Hartree & Mann, 1959). As suspending media we have used (i) the calcium-free Ringer solution previously used by Mann (1946) for studies on the metabolism of spermatozoa ('sperm-Ringer'), and (ii) the same medium with the addition of phosphate buffer, pH 7·4, which was designated 'Ringer-phosphate' by Mann & White (1957). Suspensions of erythrocytes were prepared from defibrinated horse blood. The blood was centrifuged, the plasma sucked off and the cells were washed twice with Ringer-phosphate.

We are indebted to Dr R. M. C. Dawson for a sample of lysolecithin. This had been prepared from lecithin by the action of snake venom but had been crystallized from ethanol and so was presumably free from venom (see Marples & Thompson, 1958). The absence of venom was confirmed when it was found that the lytic action of the sample towards erythrocytes (see Analytical methods below) was unchanged by a second recrystallization from ethanol at $-15°$. Palmitaldehyde and its thiosemicarbazone were obtained as previously described (Hartree & Mann, 1959).

Ether was distilled from sodium and all other solvents were redistilled before use. All solvent mixtures are specified on a volume/volume (v/v) basis.

Analytical methods

Nitrogen was determined according to Chibnall, Rees & Williams (1943) and phosphorus according to King (1932). Estimations of aldehydogenic lipids (plasmalogen plus lysoplasmalogen) and of lipid acyl ester were made as previously described (Hartree & Mann, 1959). Choline was estimated by Glick's (1944) method. Fructose was determined according to Roe (1934), after deproteinization by the $Ba(OH)_2$–$ZnSO_4$ method of Somogyi (1945). Fructolysis was followed by the method of Mann (1948). Uptakes of O_2 were measured in Barcroft differential manometers at 37°; the centre wells of the flasks contained 2·5 N-NaOH and a roll of filter paper for absorption of CO_2. Lysis of erythrocytes was measured at 20° by the spectroscopic method of Keilin & Hartree (1946).

Chromatography on silicic acid columns was carried out at 20° according to Hanahan, Dittmer & Warashina's (1957) procedure for phospholipids. The adsorbent was a mixture of silicic acid (Mallinckrodt, 100 mesh, analytical reagent, 'suitable for chromatographic analysis') and

Hyflo Supercel (Johns–Manville Co. Ltd.). Ascending chromatography on silicic acid-impregnated papers was performed as described by Marinetti & Stotz (1956) except that smaller papers were used and the duration of the runs was curtailed. The papers (Whatman no. 1) were in the form of cylinders, 16 cm. × 5·5 cm. diam., and the chromatograms were developed at 24° in 2 lb. screw-top fruit-preserving jars. The developing solvent was di*iso*butylketone–acetic acid–water (8:5:1). Since lysoplasmalogen is known to be acid-labile the chromatograms were run for only 3 hr. The resolution under these conditions was less complete than with the larger papers and longer times as specified by Marinetti & Stotz (1956). The lipids on the developed chromatograms were made visible by treatment with rhodamine 6G and examination in u.v. light (Marinetti & Stotz, 1956). Aldehydes and aldehydogenic lipids on the papers were detected as follows. After the treatment with rhodamine 6G the papers were placed for 5 min. in the magenta–bisulphite reagent of Feulgen, Boguth & Andresen (1951), washed for 5 min. in running water, placed for 5 min. in 0·5% (w/v) sodium metabisulphite in 0·1 N-HCl, washed again with water and finally soaked for 1 min. in ethanol.

Preparation of choline lysoplasmalogen (lysophosphatidal choline)

All evaporations were carried out under reduced pressure in a rotary evaporator.

A mixture of lecithin and choline plasmalogen was isolated from ox-heart muscle by Pangborn's method (see Lees, 1957). Purification was carried to the stage of treatment with $Ba(OH)_2$ and CO_2. Three hearts yielded 710 g. of acetone-dried powder from which 10·4 g. of lecithin–plasmalogen mixture was obtained. According to Rapport & Franzl (1957) this fraction contains 60% of choline plasmalogen and 40% of lecithin.

The lipid mixture was hydrolysed under conditions where only acyl-ester bonds are split (Dawson, 1954; Rapport & Lerner, 1958*a*), and treated in a manner similar to that described by Thannhauser, Boncoddo & Schmidt (1951). The lipid (5·2 g.) was dissolved in a mixture of 720 ml. of methanol and 90 ml. of water at 37° and treated with 200 ml. of warm methanolic N-NaOH. The mixture was held at 37° for 20 min. and then cooled to −50° in solid CO_2–acetone. To the cold mixture methanolic acetic acid was added dropwise, with thorough mixing until the pH fell to 6·5. About 200 ml. of normal acid was required. The solution was evaporated to a syrup, which was shaken with a mixture of 150 ml. of water, 40 g. of Na_2SO_4 and 140 ml. of chloroform–methanol (9:1). The cloudy lower layer was evaporated and the residue was dissolved in 40 ml. of the same chloroform–methanol mixture. This solution was treated with 10 vol. of acetone and left overnight at 4°. The resulting precipitate was shaken with 50 ml. of chloroform–methanol, a small insoluble residue was filtered off and the filtrate treated with 200 ml. of ether. After the mixture had been held at −15° for 4 hr. the precipitate was collected in a refrigerated centrifuge, washed with 100 ml. of cold ether and freed from residual solvent in a vacuum desiccator. The product was dissolved in 3 ml. of methanol, diluted with 10 vol. of propan-2-ol and stored overnight at −15°. A small yellow precipitate was centrifuged down in the cold and discarded. The supernatant solution was evaporated to give 614 mg. of lipid (containing 4·35% of phosphorus), which was dissolved in chloroform–methanol (1:1) to a volume of 10 ml. and stored at −15°.

Further purification was effected on a column, 28 mm. diam., containing a mixture of 36 g. of silicic acid and 18 g. of Hyflo Supercel which had been previously well washed with chloroform–methanol (1:1) and then poured into the column as a suspension in the same solvent mixture. Of the above-mentioned lipid solution, 8·9 ml. (containing 23·7 mg. of phosphorus) was added to the column, which was then developed by successive addition of the following solvents: 350 ml. of chloroform–methanol (1:1), 470 ml. of chloroform–methanol (2:5), 350 ml. of methanol, 350 ml. of methanol–water (9:1). A slight positive pressure (≯0·4 atm.) of N_2 was applied to the column to maintain a flow rate of 4–5 ml./min. The outflowing solvent was collected as 9·4 ml. fractions. The results of this fractionation in terms of phosphorus (mg./fraction) are shown in Fig. 1. Fractions 12–20 were combined and evaporated to yield a mixture of yellow gum and white solid. From fractions 32–47 a similar but much smaller residue was obtained in which the solid predominated. These two fractions were combined to yield a chloroform-soluble gum (fraction A; 34·7 mg.) and an insoluble residue which was found to be silicic acid.

The main lipid component was present in fractions 51–76, which were combined and evaporated to yield a white waxy solid. This was dissolved in 6 ml. of chloroform–methanol (1:1) and a small residue of silicic acid was centrifuged down. The clear supernatant solution was treated with 40 ml. of ether and left overnight at 5°. The resulting white precipitate was centrifuged down in the cold and washed at 5° with ether–chloroform (10:1) and then with ether. The solid was dissolved in 1·5 ml. of methanol and ether was added, at the boiling point of the mixture, until a slight opalescence persisted. The mixture was cleared by addition of 1 drop of methanol and allowed to cool to 20°. The lipid crystallized in rosettes and bundles of fine needles (Plate 1). Crystallization was completed by cooling the

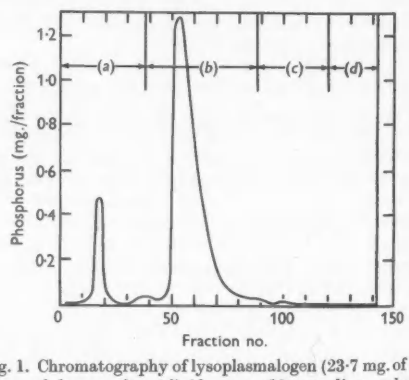
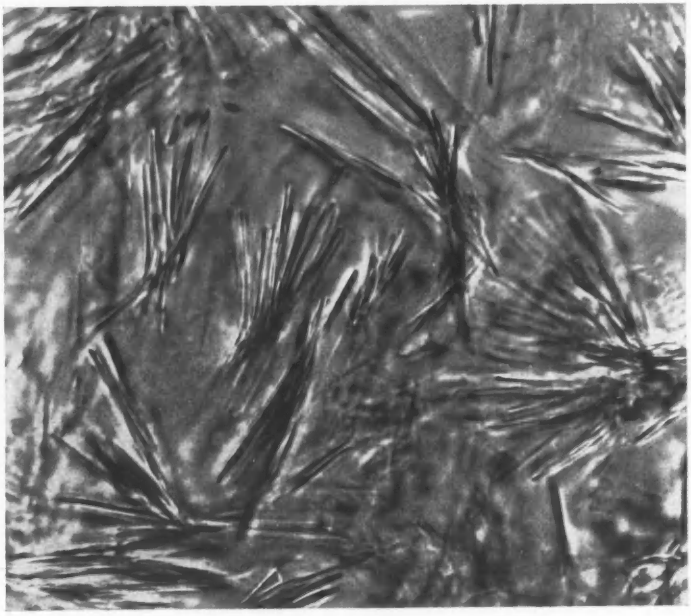


Fig. 1. Chromatography of lysoplasmalogen (23·7 mg. of P), prepared from ox-heart lipids, on an 18 mm. diam. column consisting of 36 g. of silicic acid and 18 g. of Hyflo Supercel. Solvents: (*a*) chloroform–methanol (1:1); (*b*) chloroform–methanol (2:5); (*c*) methanol; (*d*) methanol–water (9:1).



Crystalline choline lysoplasmalogen (× 1500) from methanol–ether.

mixture to $-10°$, centrifuging the mixture at the same temperature and washing the crystals with cold ether. The lipid was too hygroscopic to be obtained in the form of a powder and was therefore dissolved in dry methanol to a final volume of 10 ml. (fraction B; 190 mg.). In Table 1 are shown the analyses of fractions A and B together with theoretical values for stearal lysoplasmalogen.

Hydrogenation of lysoplasmalogen. According to Rapport *et al.* (1957), hydrogenation of plasmalogen under the conditions described by Mead & Howton (1950) results in the uptake of 1 mole of H_2, which adds on to the unsaturated ether grouping. The resulting saturated ether is acid-stable and is devoid of aldehydogenic properties. The procedure of Mead & Howton was followed except that uptake of H_2 was not measured. A solution of 38 mg. of lysoplasmalogen in 10 ml. of methanol was shaken for 2 hr. with 2 mg. of palladium–charcoal (5%, w/w) in an atmosphere of H_2. The catalyst was removed by centrifuging in the presence of a little Hyflo Supercel and the solution was cooled to $-15°$. The saturated ether was deposited as a white crystalline powder (25 mg.). From determinations of the ratio aldehyde:phosphorus it could be concluded that the lipid contained less than 1% of residual plasmalogen.

RESULTS

Comparison of the lytic activities towards erythrocytes of lysolecithin, lysoplasmalogen and hydrogenated lysoplasmalogen

The spectroscopic method for measuring rates of lysis (Keilin & Hartree, 1946) takes advantage of the fact that erythrocytes are impermeable to ferricyanide ions. Thus if ferricyanide is present in the iso-osmotic suspending medium the progress of lysis can be followed in terms of the appearance of methaemoglobin, which is formed instantaneously when the liberated oxyhaemoglobin comes into contact with ferricyanide. The ratio methaemoglobin:oxyhaemoglobin in the mixture can be determined continuously by visual spectroscopic comparison of the sample with an optical mixture of the two pigments contained separately in the compartments of a double-wedge trough.

Washed horse erythrocytes were suspended in Ringer-phosphate at a concentration of about 10^6 cells/ml., and solid $K_3Fe(CN)_6$ was added to give a concentration of 1·5 mM. This suspension was placed in both compartments of the double-wedge trough and lysolecithin in excess was added to one compartment to lyse the cells. A sufficient volume of the same suspension was placed in a flat-bottomed glass tube so that the depth of suspension equalled the optical width (2·3 cm.) of the double-wedge trough. To this end 4·05 ml. of suspension was added to a 15 mm. diam. tube. The lyso compound, dissolved in $\geqslant 0{\cdot}2$ ml. of Ringer-phosphate, was stirred into the flat-bottomed tube at zero time and the time for 80% lysis was measured with a stop-watch. The results recorded in Table 2 indicate that the three lyso compounds under test have very similar activities and that in all cases a minimum of about 5×10^7 molecules of

Table 1. *Analysis of lipid-fractions A and B from silicic acid column and theoretical figures for stearal lysoplasmalogen*

	Fraction A (m-equiv./g. of lipid)	Fraction B (%)	Fraction B (m-equiv./g. of lipid)	Theoretical values for stearal lysoplasmalogen (m-equiv./g. of lipid)
Phosphorus	0·414	5·92	1·92	1·92
Nitrogen	0·447	2·73	1·95	1·92
Fatty aldehyde	0·256	—	1·91	1·92
Choline	0·116	—	1·93	1·92
Acyl ester	0·045	—	0·02	0

Table 2. *Lytic activities of lysolecithin, lysoplasmalogen and hydrogenated lysoplasmalogen towards horse erythrocytes* ($1{\cdot}05 \times 10^6$/*ml.*) *at* 20°

Lysin	Concentration of lysin (μM)	$10^{-8} \times$ Molecules of lysin/cell	Time for 80% lysis (sec.)
Lysolecithin	24	1·36	18
	12	0·68	29
	6	0·34	No lysis in 25 min.
Lysoplasmalogen	24	1·36	20
	12	0·68	29
	6	0·34	No lysis in 25 min.
Hydrogenated lysoplasmalogen	24	1·36	20
	12	0·68	27
	6	0·34	40% lysis in 25 min. No further change at 50 min.

lysin/erythrocyte is necessary for lysis to occur at a significant rate.

Effect of lyso compounds upon the metabolism of ram spermatozoa

Effect of lysolecithin, lysoplasmalogen and hydrogenated lysoplasmalogen upon respiration. Manometer flasks received 2 ml. of suspension of washed spermatozoa ($\sim 10^9$ cells) in sperm-Ringer together with either 1 ml. of Ringer-phosphate or 1 ml. of a solution of lyso compound in the same medium. The flasks were equilibrated for 15 min. before the taps were closed.

The effects of the three lyso compounds upon the oxygen uptake of spermatozoa are shown in Fig. 2. Under the experimental conditions both lysoplasmalogen and its hydrogenated derivative were more inhibitory than lysolecithin. Thus the first two compounds were effective inhibitors at concentrations below 1 mM whereas lysolecithin had a slight stimulating effect at the same level and only became inhibitory at concentrations above 1 mM.

Effect of lysoplasmalogen on fructolysis. For this experiment 4 ml. of whole ram semen was diluted with 6 ml. of sperm-Ringer and 15 ml. of 0·5% (w/v) fructose in Ringer-phosphate. Of this suspension, 10 ml. was diluted with 5 ml. of Ringer-phosphate and a second 10 ml. with a solution of 25 mg. of lysoplasmalogen in 5 ml. of Ringer-phosphate. Two Barcroft manometers each received 3 ml. of control suspension and a further two each received 3 ml. of suspension containing lysoplasmalogen (final concentration 3 mM). During a period of 100 min. an 82% inhibition of oxygen uptake was observed. Anaerobic incubations at 37° for the same period were carried out by placing each suspension in a narrow stoppered test tube with the minimum air space. The fructose contents of the freshly made and the incubated suspensions are recorded in Table 3. Under anaerobic conditions fructolysis was inhibited by 70%. In the presence of air, the effect of lysoplasmalogen was less marked.

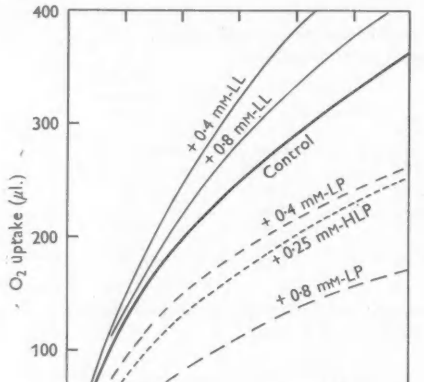


Fig. 2. Effect of lysolecithin (LL), lysoplasmalogen (LP) and hydrogenated lysoplasmalogen (HLP) on the respiration of washed ram spermatozoa (10^9 cells) suspended in a mixture of sperm-Ringer (2 ml.) and Ringer-phosphate (1 ml.).

Absence of fatty-aldehyde formation during incubation of spermatozoa with lysoplasmalogen. The lability of lysoplasmalogen in acid solution is such that paper chromatography under the conditions described by Marinetti & Stotz (1956) leads to quantitative hydrolysis and the appearance of a compact spot of free fatty aldehyde at the solvent front. Under the conditions specified for the present investigation there is considerable forward streaming of free aldehyde from the slow-moving lysoplasmalogen spot during the 3-hr. run, but no aldehyde reaches the solvent front (Fig. 3). It was thus possible to demonstrate that contact of lysoplasmalogen with ram spermatozoa does not lead to liberation of free aldehyde.

Incubation of washed spermatozoa with 8 mM-lysoplasmalogen was carried out in duplicate manometers for 2 hr. as described in the previous section on respiration of spermatozoa (see Fig. 2). Two control manometers were run simultaneously. The effect of the lyso compound was to inhibit respiration by 90%. At the end of the incubation the contents of the duplicate manometers were

Table 3. *Effect of lysoplasmalogen on fructolysis in ram semen*

The semen was diluted with 8·4 vol. of a mixture of sperm-Ringer and Ringer-phosphate containing 10 mM-fructose (see text). Lysoplasmalogen was present at a concentration of 3 mM. Aerobic experiments were carried out in Barcroft manometers; anaerobic conditions were achieved by placing the suspensions in stoppered tubes with the minimum air space. Temp., 37°.

	Fructose concentration (mM)	
	Without lysoplasmalogen	With lysoplasmalogen
Initial suspension	8·5	8·5
After aerobic incubation for 100 min.	6·2	7·3
After anaerobic incubation for 100 min.	4·8	7·3

combined to give two samples (with and without lysoplasmalogen) of 6 ml. To each was added, with shaking, 120 ml. of chloroform–methanol (2:1). The precipitate was filtered off and the filtrate shaken with 0·2 vol. of 3 mM-$MgCl_2$ (Folch, Lees & Sloane-Stanley, 1957). The lower layer was evaporated completely and the residue dissolved in 1·2 ml. of methanol. Of the two solutions thus obtained 20 μl. was spotted on to silicic acid-impregnated paper and the chromatogram was developed for 3 hr. A palmitaldehyde standard was run simultaneously.

The results obtained after staining the paper with Schiff's reagent are represented in Fig. 3. The nature of the components obtained from sperm lipid (I) has not been completely established but the spots are provisionally identified as follows: (*a*) neutral lipids and fatty acids; (*b*) free aldehyde derived from labile aldehydogenic lipids; (*c*) ethanolamine plasmalogen; (*d*) choline plasmalogen (major component); (*e*) sphingolipid plus a plasmalogen-like lipid; (*f*) inositides plus lyso compounds (including any lysoplasmalogen). Authentic palmitaldehyde (II) travelled at the solvent front. Lipids derived from the mixture of spermatozoa and lysoplasmalogen (III) gave the staining pattern characteristic of lysoplasmalogen with considerable free aldehyde streaming ahead of the lipid spot. Chromatography of a mixture of palmitaldehyde and III produced a normal aldehyde spot at the solvent front. Thus the rate of movement of free fatty aldehyde was not influenced by the presence of an excess of other lipids. Since the molar ratio lysoplasmalogen in III/palmitaldehyde in II was 12 and since no Schiff's-positive reaction was obtained at the solvent front in III it can be deduced that no appreciable formation of free aldehyde occurred before the application of spot III to the paper.

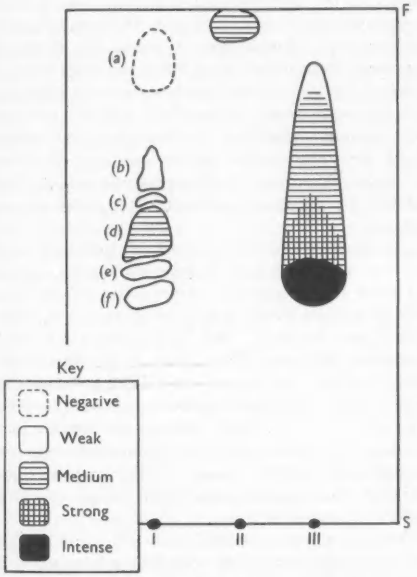


Fig. 3. Chromatograms on silicic acid-impregnated paper of palmitaldehyde and of lipids extracted from ram spermatozoa ± lysoplasmalogen. Solvent: di*iso*butylketone–acetic acid–water (8:5:1), run for 3 hr. at 24°. The washed spermatozoa had previously been incubated aerobically for 2 hr. under the conditions of the experiments in Fig. 2. I, Lipids (≡30 μl. of semen) extracted from incubated spermatozoa; II, palmitaldehyde (0·08 μmole); III, lipids extracted from spermatozoa previously incubated in the presence of 8 mM-lysoplasmalogen (0·96 μmole of lysoplasmalogen present on the paper). S, Starting line; F, solvent front. Spots were made visible by staining with Rhodamine 6G and examination under ultraviolet light. The paper was subsequently treated with Schiff's reagent to give staining reactions indicated in the key.

DISCUSSION

The exact chemical constitution of the crystalline lysoplasmalogen described in this paper remains uncertain. It is now well established that the aldehydogenic properties of plasmalogens and lysoplasmalogens derive from the presence of an acid-labile $\alpha\beta$ unsaturated ether group linking a non-polar fatty chain to a carbon atom of glycerol (Debuch, 1957; Rapport & Lerner, 1958*b*; Blietz, 1958). The ready cleavage of the unsaturated ether to yield fatty aldehyde is the basis of methods for estimation of plasmalogen. It is still a matter of dispute which hydroxyl group of glycerol is involved in ether formation (Klenk & Debuch, 1959). Before the unsaturated-ether nature of plasmalogens had been established it had been widely accepted that the acid-labile aldehydogenic group was linked at the α'-carbon atom (Klenk & Debuch, 1954). Whereas one school supports this contention by postulating an unsaturated ether at the α' position and an acyl ester at the β position of glycerol (Marinetti & Erbland, 1957; Marinetti, Erbland & Stotz, 1958*a*), other investigators adduce evidence for the reverse (Rapport *et al.* 1957; Gray, 1957). The problem has been rendered more complex by the findings (i) that at least in pig-heart muscle both isomeric plasmalogens may occur (Marinetti & Erbland, 1957; Marinetti *et al.* 1958*a*), and (ii) that the action of snake venom on lecithin can, contrary to general belief, give rise not only to β-lysolecithin but also to α'-lysolecithin (Marinetti, Erbland & Stotz, 1958*b*). It thus follows that the ability of venom to split fatty acid from plasmalogens (Rapport & Franzl, 1957; Hartree & Mann, 1959) is no longer

a satisfactory criterion for an α′-acyl-ester structure. In lysolecithin the transfer of acyl-ester linkage from the β to the α′ position takes place readily in dilute acid or in the presence of a specific enzyme (Uziel & Hanahan, 1957). Thus even when the structure of a plasmalogen has been fully established, the nature of a derived lysoplasmalogen will remain uncertain until the possibilities of β–α′ migration have been eliminated. We have found that paper chromatography of ram-sperm lipid indicates the presence in very small amount of a second plasmalogen-like component [Fig. 3 (*e*)]. It is thus possible that both plasmalogen isomers are present in ram spermatozoa.

If we are correct in suggesting (Hartree & Mann, 1959) that in the absence of fructose the plasmalogen in ram spermatozoa breaks down to provide fatty acids as a source of energy, then lysoplasmalogen should also be formed. It is remarkable that the three lyso compounds examined in the present study, despite their distinctive chemical properties, should be indistinguishable in terms of their power to lyse erythrocytes. Since lysoplasmalogen is highly active in this respect it appeared feasible to repeat with ram spermatozoa the experiments carried out by Maggio & Monroy (1955) on sea-urchin (*Arbacia lixula*) spermatozoa. It had been established earlier by Rothschild & Cleland (1952) that metabolism in sea water of spermatozoa of the sea urchin *Echinus esculentis* involves extensive breakdown of phospholipids. Maggio & Monroy (1955) found that when sea-urchin spermatozoa were suspended in sea water a substance diffused from the spermatozoa which had a lytic action upon erythrocytes. Furthermore, this lytic action became more pronounced if lipovetillin was added to the suspension. It thus seems likely that these spermatozoa contain a phospholipase A that can hydrolyse both intra- and extra-cellular phosphatides.

We carried out similar experiments with suspensions of washed ram spermatozoa in sperm-Ringer but in no case could any lytic agent be detected in the Ringer medium. This did not appear to be due to simultaneous production of a lysis inhibitor since lysolecithin and lysoplasmalogen, when added to such suspensions, exhibited their normal lytic activities. These negative results do not dismiss the possibility that lysoplasmalogen is formed when motile ram spermatozoa are stored in sugar-free media. It is known that mammalian spermatozoa are less permeable than sea-urchin spermatozoa (Mann, 1954): thus inward diffusion of lipids and outward diffusion of such metabolic products as lysophosphatides may not occur. This non-diffusibility may be due to a stronger lipid–protein binding, which, in itself, might modify or even suppress the inhibitory action of any intracellular lysoplasmalogen.

Although extracellular lysoplasmalogen is apparently not metabolized by ram spermatozoa it is still possible that the same lipid, if it is produced within the cell, may undergo further changes. It is hoped that new conditions for paper chromatography, under which lysoplasmalogen will be both stable and well separated from other lipids, will throw light on this problem. In the meantime it can be stated that there are significant decreases in the plasmalogen content of ram spermatozoa after incubation in sugar-free media (E. F. Hartree & T. Mann, unpublished results).

The toxic actions of snake and other venoms on living cells and on isolated enzymes and enzyme systems (Zeller, 1951; Braganca & Quastel, 1953) have in the main been ascribed to the presence of phospholipase A. Thus by heat-treatment of cobra and viper venoms Braganca & Quastel (1953) were able to inactivate all enzymes known to be present except phospholipase A. With such heated venoms they demonstrated that the types of enzyme system which were inhibited were those in which activity is linked with structural organization of component catalysts: soluble enzymes were mainly unaffected. The observed effects could be interpreted as an action of phospholipase A upon the lipoproteins which bind together the components of organized multi-enzyme systems.

Since lysolecithin is a product of phospholipase A activity and since it is highly surface-active it is not easy to distinguish between the effects upon cells or enzyme systems of phospholipase A, which hydrolyses lecithin, and of lysolecithin itself. Webster (1957) and Thompson & Webster (1957) associate the 'clearing action' of lysolecithin upon dispersions of brain and other tissues with its effect upon lipoproteins. This clearing action is independent of the presence of venom impurity (Habermann & Neumann, 1954) and is qualitatively indistinguishable from that of other surface-active agents.

Oxidative phosphorylation in mitochondria and in tissue dispersions is completely abolished by very low concentrations of lysolecithin (Habermann, 1954; Witter, Morrison & Shepardson, 1957); similarly the complete reaction chain for oxidation of succinate or β-oxybutyrate by oxygen is sensitive to lysolecithin but the component parts (e.g. succinic dehydrogenase, cytochrome oxidase) are not. These results suggest that the effect of lysolecithin is upon the binding structures of the complex of enzymes making up the respiratory chain rather than upon individual enzymes. Comparison of these results with those of Braganca & Quastel suggest that the action of venoms is due essentially to the lysolecithin that is released by phospholipase A action.

Whereas both snake venom and lysolecithin have powerful inhibitory actions upon respiration and motility of spermatozoa, their effects upon sperm morphology are quite distinct. Treatment of ram spermatozoa with venom in the manner previously described (Hartree & Mann, 1959) gives rise to a loosening and fragmentation of the outer cell structure and also to a separation of some of the heads from midpiece–tails. Dawson *et al.* (1957) reported that lysolecithin treatment of ram spermatozoa brings about a detachment of heads. However, with lower concentrations, we have observed neither detachment nor cellular disruption when spermatozoa are incubated at 37° with 5 mM-lysolecithin or lysoplasmalogen for as long as 2 hr. Under the same conditions, however, the inhibitory effect upon motility and respiration is apparent after very brief contact (Fig. 2).

Since both respiration and fructolysis are sensitive to lysolipids it may be assumed that the action of such compounds is not restricted to a particular enzyme or enzyme complex. It is possible that their action is primarily upon the lipoprotein of the outer cell structure, leading to impairment of selective permeability, with secondary actions upon the internal organization of the cell. The slight activating effect of lysolecithin at certain concentrations (Fig. 2) may be a resultant of the characteristic inhibitory effect of a lysolipid and the non-specific protective effect of phospholipids upon respiration and motility of spermatozoa.

SUMMARY

1. A choline-based lysoplasmalogen has been prepared in crystalline form from an alkaline hydrolysate of the lecithin fraction from ox heart. The ratio phosphorus:choline:fatty aldehyde is 1:1:1.

2. Lysoplasmalogen, hydrogenated lysoplasmalogen and lysolecithin are quantitatively equivalent as lysins for horse erythrocytes.

3. The respiration of ram-sperm suspensions (3×10^8 cells/ml.) is inhibited by lysolecithin only at concentrations greater than 1 mM. The other two lyso compounds are effective inhibitors at lower concentrations. Fructolysis in ram semen is also inhibited by lysoplasmalogen.

4. No free fatty aldehyde is formed when lysoplasmalogen is incubated with ram spermatozoa.

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REFERENCES

Blietz, R. J. (1958). *Hoppe-Seyl. Z.* **310**, 120.
Braganca, B. M. & Quastel, J. H. (1953). *Biochem. J.* **53**, 88.
Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 354.
Dawson, R. M. C. (1954). *Biochim. biophys. Acta*, **14**, 374.
Dawson, R. M. C., Mann, T. & White, I. G. (1957). *Biochem. J.* **65**, 627.
Debuch, H. (1957). *Biochem. J.* **67**, 27P.
Feulgen, R. & Bersin, T. (1939). *Hoppe-Seyl. Z.* **260**, 217.
Feulgen, R., Boguth, W. & Andresen, G. (1951). *Hoppe-Seyl. Z.* **287**, 90.
Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957). *J. biol. Chem.* **226**, 497.
Glick, D. (1944). *J. biol. Chem.* **156**, 643.
Gray, G. M. (1957). *Biochem. J.* **67**, 26P.
Habermann, E. (1954). *Naturwissenschaften*, **41**, 429.
Habermann, E. & Neumann, W. (1954). *Hoppe-Seyl. Z.* **297**, 179.
Hanahan, D. J., Dittmer, J. C. & Warashina, E. (1957). *J. biol. Chem.* **228**, 685.
Hartree, E. F. & Mann, T. (1958). *Biochem. J.* **69**, 50P.
Hartree, E. F. & Mann, T. (1959). *Biochem. J.* **71**, 423.
Keilin, D. & Hartree, E. F. (1946). *Nature, Lond.*, **157**, 210.
King, E. J. (1932). *Biochem. J.* **26**, 292.
Klenk, E. & Debuch, H. (1954). *Hoppe-Seyl. Z.* **296**, 179.
Klenk, E. & Debuch, H. (1959). *Annu. Rev. Biochem.* **28**, 39.
Lardy, H. A. & Phillips, P. H. (1941*a*). *Amer. J. Physiol.* **133**, 602.
Lardy, H. A. & Phillips, P. H. (1941*b*). *Amer. J. Physiol.* **134**, 542.
Lees, M. B. (1957). *Methods in Enzymology*, vol. 3, p. 334. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Lovern, J. A., Olley, J., Hartree, E. F. & Mann, T. (1957). *Biochem. J.* **67**, 630.
Maggio, R. & Monroy, A. (1955). *Exp. Cell Res.* **8**, 240.
Mann, T. (1946). *Biochem. J.* **40**, 481.
Mann, T. (1948). *J. agric. Sci.* **38**, 323.
Mann, T. (1954). *The Biochemistry of Semen.* London: Methuen.
Mann, T. & White, I. G. (1957). *Biochem. J.* **65**, 634.
Marinetti, G. V. & Erbland, J. (1957). *Biochim. biophys. Acta*, **26**, 429.
Marinetti, G. V., Erbland, J. & Stotz, E. (1958*a*). *J. Amer. chem. Soc.* **80**, 1624.
Marinetti, G. V., Erbland, J. & Stotz, E. (1958*b*). *Biochim. biophys. Acta*, **33**, 403.
Marinetti, G. V. & Stotz, E. (1956). *Biochim. biophys. Acta*, **30**, 187.
Marples, E. A. & Thompson, R. H. S. (1958). *Biochim. biophys. Acta*, **30**, 187.
Mead, J. F. & Howton, D. R. (1950). *Analyt. Chem.* **22**, 1204.
Rapport, M. L. & Franzl, R. E. (1957). *J. biol. Chem.* **225**, 851.
Rapport, M. M. & Lerner, B. (1958*a*). *J. biol. Chem.* **232**, 63.
Rapport, M. M. & Lerner, B. (1958*b*). *Biochim. biophys. Acta*, **33**, 319.
Rapport, M. M., Lerner, B., Alonzo, N. & Franzl, R. E. (1957). *J. biol. Chem.* **225**, 859.

Roe, J. H. (1934). *J. biol. Chem.* **107**, 15.
Rothschild (Lord) & Cleland, K. W. (1952). *J. exp. Biol.* **29**, 66.
Somogyi, M. (1945). *J. biol. Chem.* **160**, 69.
Thannhauser, S. J., Boncoddo, N. F. & Schmidt, G. (1951). *J. biol. Chem.* **188**, 417.
Thompson, R. H. S. & Webster, G. R. (1957). *Biochem. J.* **67**, 31P.
Uziel, M. & Hanahan, D. J. (1957). *J. biol. Chem.* **226**, 789.
Webster, G. R. (1957). *Nature, Lond.*, **180**, 660.
Witter, R. F., Morrison, A. & Shepardson, G. R. (1957). *Biochim. biophys. Acta*, **26**, 121.
Zeller, E. A. (1951). In *The Enzymes*, 1, part 2, p. 987. Ed. by Sumner, J. B. & Myrbäck, K. New York: Academic Press Inc.

Biochem. J. (1960) **75**, 258

Lactic Dehydrogenase and Cytochrome b_2 of Baker's Yeast

THE DEOXYRIBOSE POLYNUCLEOTIDE COMPONENT AND THE PHYSICOCHEMICAL PROPERTIES OF THE CRYSTALLINE ENZYME

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The absorption spectrum of reduced and oxidized crystalline cytochrome b_2 (Appleby & Morton, 1954, 1959*a*, *b*) shows a pronounced peak at about 265 mμ. It has been found that this absorption band is mainly due to a deoxyribose polynucleotide which is intimately associated with the enzyme as purified by the method of Appleby & Morton (1959*a*). This paper describes the chemical composition of this component and the nature of its association with the crystalline enzyme.

Electrophoresis and sedimentation studies of the crystalline cytochrome b_2 revealed only one protein component. The separation of nucleotide-depleted enzyme from intact enzyme by electrophoresis is described. The results confirm that yeast lactic dehydrogenase is identical with cytochrome b_2 and is a protein with both haem and flavin prosthetic groups (Appleby & Morton, 1954).

This work was reported by us at the meeting of the Australian and New Zealand Association for the Advancement of Science in Melbourne, 1955. It has been described by Appleby (1957), and brief accounts of some aspects have been published (Appleby & Morton, 1954; Morton, 1955, 1958).

MATERIALS

Crystalline yeast lactic dehydrogenase (*cytochrome* b_2). This was prepared as described by Appleby & Morton (1959*a*). Unless otherwise stated, the enzyme was recrystallized several times from a solution of the combined first crystals from a number of preparations.

Deoxyribonucleic acid and deoxyribonucleotides. Polymerized calf-thymus deoxyribonucleic acid (DNA) containing 8·2% of P was kindly supplied by Mr G. L. Ada, and chromatographically pure 5′-deoxyribonucleotides were obtained from the California Foundation for Biochemical Research.

Ribonucleic acid and ribonucleotides. Yeast ribonucleic acid (RNA) (British Drug Houses Ltd.) and chromatographically pure 5′-ribonucleotides (Sigma Chemical Co., St Louis, Mo., U.S.A.) were used.

Alkaline phosphomonoesterase. The purified enzyme from calf intestinal mucosa (Morton, 1954) was used.

Deoxyribonuclease and ribonuclease. Crystalline preparations from pancreas were obtained from Worthington Biochemical Corp., U.S.A.

Bovine serum albumin. Crystalline protein (The Armour Laboratories, U.S.A.) was used.

METHODS

Estimation of enzymic activity. The units of activity and the assay procedures are given by Appleby & Morton (1959*a*).

Chemical determinations

Phosphate. For inorganic phosphate, the procedure of Weil-Malherbe & Green (1951) was modified so that one-third of the amounts of all reagents were used in a final volume of 3·1 ml. With these volumes it was found necessary to shake for 1 min. instead of 15 sec. E was read at 730 mμ.

For total phosphate the sample was digested for 10 min. with 0·12 ml. of 10N-H_2SO_4 and 0·05 ml. of 72% (v/v) perchloric acid, the digest was diluted to 1·5 ml. with water, hydrolysed for 10 min. at 100° and cooled. The inorganic phosphate was then estimated, acid being omitted from the molybdate reagent.

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Protein nitrogen. Approx. 1 mg. of protein was digested for 18 hr. with 0·2 ml. of 50 % (v/v) H_2SO_4 containing copper selenite. After cooling, 3 ml. of water and then 3 ml. of Nessler's reagent (King, 1951) were added with rapid mixing. After 15 min. E was read at 480 mμ. Serum albumin (16·07 % of N; Tristram, 1953) was used as a standard.

Protein. The method of Lowry, Rosebrough, Farr & Randall (1951) was used as previously described (Appleby & Morton, 1959*a*).

Ribose. The orcinol procedure as described by Hurlbert, Schmitz, Brumm & Potter (1954) was used except that the tubes were heated for 18 min. for colour development. E was read at 660 mμ. With ribose, adenosine and 3′-adenosine monophosphate, the observed ϵ was 24×10^3, and with 5′-adenosine monophosphate and adenosine triphosphate it was $32{\cdot}5 \times 10^3$.

Deoxyribose. The method of Ceriotti (1952) was used, except that the chloroform was purified as described by Martin & Morton (1956). E was measured at 492 mμ. With calf-thymus DNA as a standard, E was $12{\cdot}2 \times 10^3$ for a solution containing M-DNA phosphorus. With deoxyribonucleoside-5′-monophosphates, the following ϵ values were obtained: deoxyadenylic acid, $12{\cdot}8 \times 10^3$; deoxyguanylic acid, $12{\cdot}2 \times 10^3$; deoxycytidylic acid, $1{\cdot}6 \times 10^3$; thymidylic acid, $7{\cdot}4 \times 10^3$.

Purines and pyrimidines. Whatman no. 1 filter papers were washed according to Hanes, Hird & Isherwood (1952), the treatment with calcium acetate being omitted. The general procedures described by Markham & Smith (1949) and by Markham (1955) were followed. The bases were separated by descending chromatography according to Wyatt (1955). The method of detection, elution and estimation of the separated bases has been described by Martin & Morton (1956).

Physicochemical methods

Electrophoresis. Measurements were carried out by J. Pye, Walter and Eliza Hall Institute, Melbourne, with a Tiselius-type moving-boundary electrophoresis apparatus. A cylindrical-lens system was used for detecting the protein and a mechanically driven syringe for compensating boundaries.

Enzyme solutions were dialysed against the buffer for 10–40 hr. and then transferred to the electrophoresis cell (capacity approx. 2 ml.). Electrophoresis was carried out at 2·5°. Photographs were taken with monochromatic light (546 mμ).

Sedimentation studies. The analyses were carried out with a Phywe ultracentrifuge by I. J. O'Donnell and E. F. Woods, Division of Protein Chemistry, Wool Textile Research Laboratories, C.S.I.R.O., Melbourne. The enzyme was dialysed against the buffer for about 14 hr. and sedimentation was carried out at 15° and at 50 000 rev./min. (approx. 182 000 g). Monochromatic light (546 mμ) was used for photography.

pH. Unless otherwise stated, this was measured at about 20° with a glass electrode.

Spectrophotometry. Measurements were made with a Beckman spectrophotometer, model DU, calibrated as previously described (Appleby & Morton, 1959*a*).

Dialysis. Unless otherwise stated, all dialysis was carried out anaerobically at 0°, air being displaced by oxygen-free nitrogen. The contents of the cellulose tubing, and the diffusate, were frequently mixed.

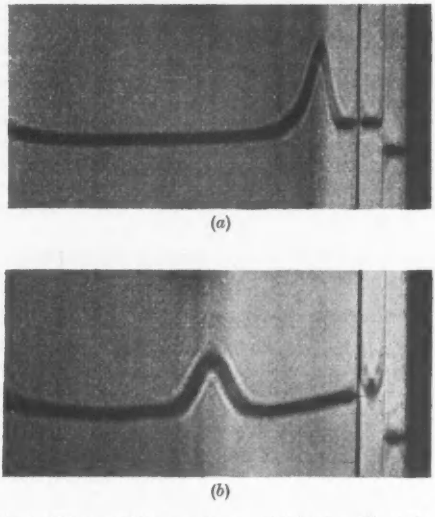
RESULTS

Physicochemical investigations

Sedimentation studies. Cytochrome b_2 prepared in the absence of ethylenediaminetetra-acetate (EDTA) and recrystallized once was dissolved (4·6 mg./ml.) in a solution of 0·15 M-NaCl and 0·4 M-sodium lactate at pH 6·8 and at 0°. In the ultracentrifuge a single component was observed which spread very little during sedimentation (Fig. 1). The boundary of the pink cytochrome, which strongly absorbed the green light used for photography, corresponded exactly with the single peak (Fig. 1).

Similar results were obtained with twice-recrystallized cytochrome b_2 prepared from a different strain of baker's yeast and with EDTA used as a protective agent (Appleby & Morton, 1959*a*) during purification. The sedimentation coefficients ($S_{20,\,w}$) were 7·77 s and 7·70 s respectively for the two analyses.

Electrophoresis studies. Analyses were carried out at pH 7·05, 6·65 and 8·8. The concentration of enzyme at pH 7·05 (3 mg./ml.) and 6·65 (2·2 mg./ml.) was lower than desirable owing to the limited solubility of the enzyme at the highest acceptable ionic strength (I 0·25) at these pH values.



(*a*)

(*b*)

Fig. 1. Ultracentrifuge patterns obtained with twice-crystallized cytochrome b_2 (4·6 mg./ml.) in 0·15 M-NaCl–0·4 M-sodium lactate, pH 6·8. Sedimentation (right to left) at 50 000 rev./min. at 15° for 20 min. (*a*) and 70 min. (*b*).

At pH 7·05 a small component appeared within 30 min. of commencement and moved rapidly towards the anode (Fig. 2). The major component moved slowly towards the anode. It remained symmetrical for 205 min. but some asymmetry was evident after 293 min. (Fig. 2). The boundary of the pink cytochrome corresponded exactly with the major component.

Similar results were obtained at pH 6·65, in 0·04 M-sodium pyrophosphate–0·06 M-lactic acid, I 0·25; field strength was 4·07 v/cm. for 325 min.

At pH 8·8 a relatively large component (corresponding to about 6 % of the area of the main peak) appeared within 20 min. and moved rapidly towards the anode (Fig. 3). At 195 min. a second peak appeared just ahead of the main pink protein boundary. At 269 min., with the aid of a black-

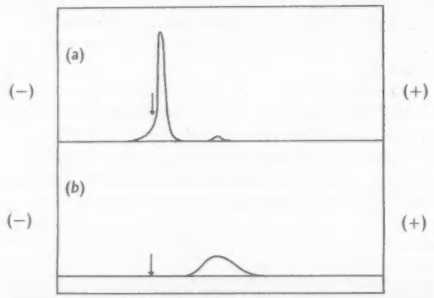


Fig. 2. Tracings of electrophoresis patterns of crystalline cytochrome b_2 (3 mg./ml.) in 0·1 M-sodium phosphate–0·05 M-sodium lactate (I 0·25, pH 7·05; field strength 3·40 v/cm.; temp. 2·5°). Ascending boundaries at 39 min. (*a*) and 293 min. (*b*). The initial boundary position is marked by the arrow.

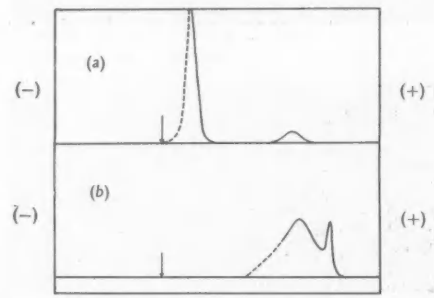


Fig. 3. Tracings of electrophoresis patterns of crystalline cytochrome b_2 (10·3 mg./ml.) in 0·1 M-sodium lactate–0·015 M-sodium pyrophosphate (I 0·25, pH 8·8; field strength 4·34 v/cm.; temp. 2·5°). Ascending boundaries at 50 min. (*a*) and at 269 min. (*b*). The initial boundary position is marked by the arrow.

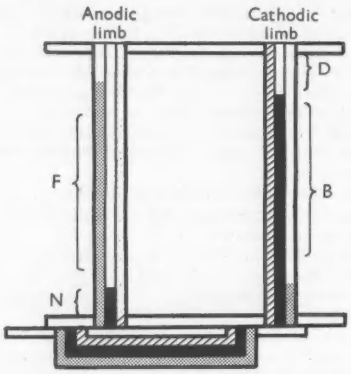


Fig. 4. Diagram showing the positions (after compensation of boundaries) of the various fractions (F, N, D and B) obtained by electrophoresis at pH 8·8 (see Fig. 3) for 304 min. Composition of the various fractions is represented by: ▤, deoxyribose polynucleotide; ■, intact enzyme (deoxyribonucleoprotein); ▨, nucleotide-depleted enzyme.

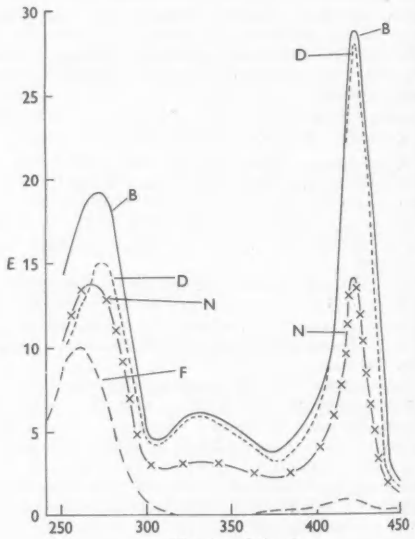


Fig. 5. Absorption spectra of fractions (see Fig. 4) obtained by electrophoresis of crystalline cytochrome b_2 at pH 8·8. Where necessary, dilutions were made in 0·5 M-sodium lactate, pH 6·8. Extinction values are given for the undiluted solutions. Some dilution with buffer occurred during removal of fractions from the electrophoresis cell.

paper mask to reduce exposure on the main peak, a photograph clearly showed the double peak on the ascending boundary (Fig. 3) and an asymmetric peak on the descending boundary.

After 304 min., when the resolution of the two peaks had increased further, electrophoresis was stopped. The boundaries were compensated to the positions shown in Fig. 4 and then four fractions (F, N, B and D) were removed from the cell. The absorption spectra of the fractions are shown in Fig. 5.

Fraction F showed a faint-yellow colour and a characteristic flavin fluorescence but, even after oxidation, no flavin absorption bands could be detected near 375 or 450 mμ (Fig. 5).

Paper electrophoresis of a portion of this fraction was carried out at 400v for 18 hr. in 0·1M-sodium phosphate–potassium phosphate buffer, pH 6·8. A large amount of material which absorbed at 254 mμ remained at the origin; it was not eluted by treatment with 0·1N-HCl at room temperature, whereas mononucleotides are readily eluted by this treatment. This material was partly eluted by shaking overnight with 0·1N-HCl at 30°. The eluate showed an absorption spectrum typical of a nucleotide ($\lambda_{max.}$, 262 mμ; $\lambda_{min.}$, 230 mμ; $E_{230\ m\mu}/E_{262\ m\mu}$, 0·21).

Fig. 5 and Table 1 show that the absorption spectrum of fraction N was essentially similar to that of the original enzyme, whereas fractions B and D (see Fig. 4) respectively contained a decreasing amount of nucleotide material. These observations showed that fraction F contained a nucleotide which had separated from a portion of the enzyme.

Polynucleotide component of cytochrome b_2

Phosphorus analyses. The total P of the crystalline enzyme, determined by Dr K. W. Zimmerman (Microanalytical Laboratory, University of Melbourne) was 0·56%; there was no inorganic P. Since cytochrome b_2 contains 0·77% of protohaem and equimolecular amounts of protohaem and riboflavin phosphate (Appleby & Morton, 1954, 1959*b*), there are approx. 15 g.atoms of total P, comprising 14 g.atoms of nucleotide P and 1 g.atom of riboflavin P/mole of haem.

Nucleotide analyses. In preliminary studies, denaturation of the enzyme and extraction of flavin with methanol (66%, v/v) at 38° was found to be unsatisfactory, as the supernatant obtained after centrifuging at 25 000 *g* contained some denatured haemoprotein and material other than flavin which absorbed u.v. light. At 0°, acidification of an enzyme solution with HCl caused maximum turbidity at pH 4·0 but, on further addition of HCl to 0·25N, a clear-yellow solution was obtained. Subsequently therefore the riboflavin phosphate was extracted and the protein precipitated as follows.

Solutions of cytochrome b_2 were brought to 0·25N with perchloric acid at 0° to remove riboflavin phosphate (Appleby & Morton, 1954, 1959*b*) and the precipitates were re-extracted with 0·25N-perchloric acid at 0°. The precipitates so obtained were then extracted with 0·5N-perchloric acid at 80° for 1 hr. The colourless supernatants had absorption spectra characteristic of nucleotides ($\lambda_{max.}$, 265 mμ; $\lambda_{min.}$, 234–240 mμ) and contained no flavin. Assuming ϵ $10{\cdot}0 \times 10^3$ at 265 mμ for the nucleotide mixture in perchloric acid (Beavan, Holiday & Johnson, 1955), the results of several such studies indicated that the enzyme contains between 9 and 14 nucleotide units/mole of haem.

Extraction of nucleotides with hot HCl instead of perchloric acid was then investigated. Cytochrome b_2 (5·6 mg.) was suspended in 2 ml. of 0·5N-HCl at 20°. After heating at 80° for 30 min., a flocculent brown precipitate and a pale-yellow supernatant were obtained. The precipitate was collected by centrifuging and washed by resuspending in 0·5N-HCl at 80°; E at 265 mμ of the combined supernatants (2·6 ml.) was 5·52. If all of the flavin of the enzyme was degraded to lumichrome ($\epsilon_{265\ m\mu}$ 31×10^3; McNutt, 1954) then E due to nucleotide

Table 1. *Properties of the fractions obtained by electrophoresis of crystalline cytochrome* b_2 *at* pH 8·8

Recrystallized cytochrome b_2 was dialysed anaerobically (see Methods section) for 10 hr. at pH 8·8. Electrophoresis was then carried out (under the conditions given in Fig. 3) for 304 min. The positions of the various fractions after electrophoresis are shown in Fig. 4. Protein was estimated according to Lowry, Rosebrough, Farr & Randall (1951) (see Methods section). $E_{u.v.}$ and E_{Soret} refer to the maximum extinctions determined at the appropriate wavelengths shown under $\lambda_{max.}$.

Fraction	Concn. of protein (mg./ml.)	$\lambda_{max.}$ (mμ) U.v. band	$\lambda_{max.}$ (mμ) Soret band	$\frac{E_{u.v.}}{E_{Soret}}$	$\frac{E_{u.v.}}{\text{Protein (mg./ml.)}}$	$\frac{E_{Soret}}{\text{Protein (mg./ml.)}}$
F	0·19	260	422·5	17·8	52·8	2·96
N	5·0	265–270	422	0·96	2·70	2·82
B	9·9	272	423	0·67	1·94	2·91
D	8·95	272	423	0·54	1·68	3·12
Enzyme*	10·3	266	423	0·93	2·74	2·96

* After dialysis at pH 8·8 and before electrophoresis.

only would be 4·68. Assuming that $\epsilon_{265\,m\mu}$ for degraded nucleotides is $10{\cdot}0 \times 10^3$ (Beavan *et al.* 1955), then these results suggest that the enzyme contains about 17 moles of nucleotide/mole of haem. The extract contained 15·6 g.atoms of P and the residue 1·0 g.atom of P/mole of haem in the original enzyme.

Identification and estimation of nucleotide bases. In a preliminary experiment, riboflavin phosphate was extracted from cytochrome b_2 with 0·25 N-perchloric acid at 0°, and nucleotides were extracted from the residue with 0·5 N-perchloric acid at 80° (see above). The extract so obtained was brought to 72% (v/v) with perchloric acid and digested at 100° for 30 min., cooled and adjusted to pH 4·5 with KOH. The potassium perchlorate so obtained was removed by centrifuging. The bases in the digest were separated by paper chromatography (see the Methods section) and identified by comparison with those in similar digests of RNA and DNA applied to the same paper. Adenine, guanine, cytosine and thymine were detected but interference by compounds absorbing non-specifically prevented identification of the pyrimidines with certainty.

The nucleotides were then extracted from a solution of oxidized cytochrome b_2 (containing 21·6 μg. of organic P) with 0·5 N-HCl at 80°. A portion (1·84 ml.) of the clear-yellow supernatant (2·04 ml.) was freeze-dried and dissolved in 0·6 ml. of formic acid (88%, v/v) and digested at 175° for 1 hr. (Wyatt, 1955) in ampoules sealed under formic acid vapour and containing equal gas and liquid volumes. After drying over NaOH, the hydrolysate was dissolved in 39 μl. of N-HCl. Samples of DNA (2 mg.) and of RNA (2 mg.) were each hydrolysed in 1 ml. of formic acid under the same conditions. For chromatography, 12·5 μl. portions of the enzyme hydrolysate and 6·4 μl. portions of the other hydrolysates were applied to separate strips (about 2 cm. wide) from the one sheet of paper, and the chromatogram was developed with the propan-2-ol–HCl solvent of Wyatt (1955).

Comparison of the u.v.-absorbing spots in the hydrolysates of the enzyme with those of hydrolysates of RNA and DNA, and with published R_F values of purines and pyrimidines (Wyatt, 1955), shows that the enzyme contains only guanine, adenine, cytosine and thymine. Uracil was not detected.

When irradiated at about 360 mμ the chromatogram showed a faint-blue fluorescence close to the expected position of uracil (R_F 0·63) and a faint-yellow–green fluorescence overlying the position of cytosine. These fluorescent spots were probably degradation products of riboflavin phosphate.

With similar eluates from the appropriate portions of the blank strip as references, the absorption spectra of eluates of each of the u.v.-absorbing spots in 0·1 N-HCl were determined. The results (Table 2) confirm that the enzyme contains adenine, guanine, cytosine and thymine only. No u.v.-absorbing material was detected in the eluate of the area of paper where uracil, if present in the enzyme, would be expected to occur. The agreement of the absorption spectra of the eluted bases with published figures was good for all except cytosine (see Table 2); $\lambda_{max.}$ for this was at 272 mμ

Table 2. *Estimation of nucleotide bases in crystalline cytochrome* b_2

The bases were separated from formic acid hydrolysates of recrystallized cytochrome b_2 and calf-thymus deoxyribonucleic acid by paper chromatography, and eluted with 0·1 N-HCl as described in the text; the final volume was 1·2 ml. In each case measurements were made against an eluate from an appropriate portion of a blank chromatogram. The literature values are from Beavan, Holiday & Johnson (1955) and are given for the bases at pH 1·0 and at mM concn.

		Spectrophotometric measurements					Amount (μmole) from chromatogram of	
Base	Source	λ_{max} (mμ)	E_{max}	$\lambda_{min.}$ (mμ)	$E_{min.}$	$\frac{E_{min.}}{E_{max.}}$	DNA	Enzyme
Adenine	Literature	262·5	13·15	229	2·25	0·171	—	—
	DNA	262	0·82	229	0·16	0·195	0·075	—
	Enzyme	262	0·76	229·5	0·207	0·272	—	0·069
Guanine	Literature	248·5	11·4	224	3·55	0·31	—	—
	DNA	248·5	0·613	224·5	0·292	0·476	0·065	—
	Enzyme	249·5	0·213	226·5	0·113	0·530	—	0·023
Cytosine	Literature	276	10·0	238·5	1·2	0·120	—	—
	DNA	274	0·518	239·5	0·109	0·210	0·062	—
	Enzyme	272	0·210	239	0·065	0·310	—	0·025
Thymine	Literature	264·5	7·89	233·5	1·9	0·241	—	—
	DNA	265	0·503	233	0·126	0·250	0·077	—
	Enzyme	264·5	0·363	234	0·118	0·326	—	0·055
		Total bases (μmole)			—	—	0·279	0·172
		Total P (μmole)			—	—	0·303	0·189
		Recovery (%)			—	—	92	91

in the enzyme hydrolysate and at 274 mμ in the DNA hydrolysate, whereas pure cytosine has $\lambda_{max.}$ at 276 mμ (Beavan *et al.* 1955). The shift in $\lambda_{max.}$ is probably due to traces of the compound which had a faint-yellow fluorescence and which ran close to the position of cytosine; it is not due to the presence of either 5-methylcytosine or of 5-hydroxymethylcytosine, for which, at pH 1·0, the values of $\lambda_{max.}$ are 283·5 and 279 mμ respectively (Beavan *et al.* 1955). It is nevertheless possible that very small amounts of these compounds may occur in the enzyme.

The amount of each base in the eluate was estimated from the E at $\lambda_{max.}$ and the extinction coefficients of Beavan *et al.* (1955). Assuming that the original extract of the enzyme contained 20·3 μg. of nucleotide P and 1·3 μg. of P due to riboflavin phosphate, the amount of nucleotide P applied to the chromatogram was estimated as 0·189 μmole (i.e. $20{\cdot}3 \times 1{\cdot}84 \times 12{\cdot}5/2{\cdot}04 \times 39 \times 31$). Thus the calculated recovery of the bases of the enzyme hydrolysate was 91 % (Table 2). The results for the base composition of calf-thymus DNA obtained at the same time (Table 2) agree well with those of Chargaff & Lipshitz (1953) for ox-thymus DNA.

The phosphorus and nucleotide analyses indicate that the enzyme contains approx. 15 nucleotide residues/mole of haem. From the relative proportions of each base in the enzyme hydrolysate, the number of residues of each nucleotide/mole of haem have been calculated and are shown in Table 3.

Identification and estimation of the nucleotide sugar. The solution obtained by extraction of nucleotides from cytochrome b_2 with 0·5 N-perchloric acid at 80° (described above) was used. With the orcinol method of Hurlbert *et al.* (1954), a brown solution was obtained, whereas lime-green solutions were obtained with yeast RNA and with ribose nucleotides similarly treated at the same time. The absorption spectra of the brown and of the green solutions had no similarity to one another.

With the Ceriotti (1952) method for deoxyribose, the nucleotide extract gave a yellow solution, the absorption spectrum of which was identical with that of a solution obtained from DNA treated similarly. From E at $\lambda_{max.}$ (492 mμ) and with DNA as a standard, it was calculated that the original enzyme solution, which contained 0·065 mM-enzyme-bound haem, also contained 0·766 mM-deoxyribose.

The sugar was also identified by paper chromatography. A solution of twice-crystallized cytochrome b_2 was dialysed for further crystallization as described by Appleby & Morton (1959*a*). After collection of the crystals, the remaining mother liquor (which contained some uncrystallized enzyme) was brought to 0·25 N-perchloric acid at 0° to remove flavin. The nucleotides were then extracted from the protein precipitate with 0·5 N-perchloric acid at 80° (described above). The extract, containing about 350 μg. of nucleotide, was freeze-dried. The product was dissolved in 0·1 ml. of diethylbarbiturate–HCl buffer, pH 6·9, containing 0·01 M-$MgSO_4$. Small portions of pancreatic ribonuclease and deoxyribonuclease and of alkaline phosphatase were added. Samples of DNA and of RNA were similarly treated. The mixtures were incubated for about 14 hr. at 18° and the resultant mononucleotides were hydrolysed (and proteins were precipitated) as described by Chargaff, Vischer, Doniger, Green & Misani (1949) to obtain the free sugars. Paper chromatography of the sugars was carried out according to Partridge (1948) and development was with butanol–ethanol–water (45:5:49). Papers were sprayed first with periodate and then with *p*-nitroaniline (Edward & Waldron, 1952). This test is specific for deoxypentose. A yellow spot (R_F 0·35) appeared in the DNA hydrolysate and in the enzyme hydrolysate, but not in the RNA hydrolysate. Thus the nucleotide associated with cytochrome b_2 contains a sugar apparently identical with 2-deoxyribose, the characteristic sugar of thymus DNA (Levene & Mori, 1929).

Table 3. *Base composition of the deoxyribose polynucleotide of crystalline cytochrome* b_2

The analyses were carried out on recrystallized cytochrome b_2 as described in the text and for Table 2.

Base	Moles/mole of haem: Found	Moles/mole of haem: Probable no.	Moles/100 g.atoms of P
Adenine	5·8	6	40·0
Guanine	1·9	2	13·2
Cytosine	2·1	2	14·6
Thymine	4·6	5	32·0

$$\frac{\text{Total purine (moles)}}{\text{Total pyrimidine (moles)}} : 1{\cdot}14$$

$$\frac{\text{Adenine + thymine (moles)}}{\text{Guanine + cytosine (moles)}} : 2{\cdot}60$$

Separation of cytochrome b_2 *from ribose polynucleotides during crystallization*

The finding of thymine and deoxyribose in the nucleotide associated with cytochrome b_2 and complete absence of uracil and ribose was not expected. Therefore quantitative sugar analyses were carried out on the mother liquors obtained after first crystallization and subsequent recrystallization of a large batch of enzyme. The results are shown in Table 4.

The mother liquor after first crystallization contained a ribose polynucleotide (presumably RNA),

Table 4. *Nucleotide sugars in the mother liquors remaining after crystallization of cytochrome* b_2

Cytochrome b_2 was prepared according to Appleby & Morton (1959*a*). The crystals were dissolved in 0·5 M-sodium lactate–0·1 mM-EDTA and recrystallized. Ribose and deoxyribose were estimated (see Methods section) in the mother liquor at each successive crystallization. At stage 1, before crystallization the solution contained cytochrome b_2 equivalent to 0·13 mM-deoxyribose.

Stage	Vol. (ml.)	Concn. of sugar (mM)	
		Ribose	Deoxyribose
1	400	32·4	1·6
2	100	0·007	0·082
3	95	0·002	0·065
4	10	0	0·088

equivalent to 32·4 mM-sugar, but the deoxyribose polynucleotide was only equivalent to 1·6 mM-sugar. The cytochrome b_2 crystallized from this mother liquor contained polynucleotide equivalent to 0·13 mM-deoxyribose in the mother liquor. Hence there is a specific association of the deoxyribonucleotide with crystalline cytochrome b_2. After recrystallization no ribonucleotide could be detected in the crystalline cytochrome b_2.

Dissociation of the deoxyribose polynucleotide component from the enzyme

Separation by electrophoresis. The separation of the polymerized deoxyribonucleotide in fraction F by electrophoresis at pH 8·8 has been described. As shown in Table 1, for fraction F the ratio of $E_{a.v.}$/protein concn. (mg./ml.) was 52·8 compared with 2·74 in the crystalline cytochrome b_2.

Fractionation with ammonium sulphate. After dialysis of crystallized cytochrome b_2 against 0·63-saturated $(NH_4)_2SO_4$ containing 0·5 M-sodium lactate and 0·1 mM-EDTA at pH 6·8, a heavy amorphous pink precipitate appeared, whereas a crystalline precipitate was obtained by dialysis against 0·05 M-sodium lactate–0·1 mM-EDTA, pH 6·8. The ratios of $E_{264\ m\mu}/E_{423\ m\mu}$ for the crystalline and amorphous precipitates were 0·96 and 0·67 respectively. The supernatant from the amorphous precipitate was enriched in nucleotide, the ratio $E_{262\ m\mu}/E_{423\ m\mu}$ being 1·97.

In another experiment, the amorphous precipitate obtained by dialysis of cytochrome b_2 against 0·63-saturated $(NH_4)_2SO_4$ containing 0·14 M-sodium lactate and 0·07 mM-EDTA at pH 6·0, when dissolved in 0·5 M-sodium lactate–0·1 mM-EDTA, pH 6·8, had 93% of the original lactic-dehydrogenase activity of the solution. The ratio $E_{268\ m\mu}/E_{423\ m\mu}$ was 0·54, compared with 1·06 for the crystalline enzyme. The supernatant from the amorphous precipitate contained 5% of the original enzymic activity; the ratio $E_{261\ m\mu}/E_{423\ m\mu}$ was 7·60, and the

Table 5. *Effects of deoxyribose polynucleotide component on the properties of crystalline cytochrome* b_2

At stage 1, two separate sacs of cytochrome b_2 (0·5 ml.) were dialysed against 0·7-saturated $(NH_4)_2SO_4$ containing 0·1 M-sodium lactate and 0·02 mM-EDTA, pH 5·9, at 0° to separate the polynucleotide. At stage 2, the contents of one (unopened) dialysis sac were mixed and then dialysed as for enzyme crystallization. The precipitated nucleotide-depleted enzyme was collected from the contents of the other dialysis sac by centrifuging, dissolved in 0·5 M-sodium lactate–0·1 mM-EDTA, pH 6·8, and then also dialysed as for enzyme crystallization. Lactic-dehydrogenase activities are expressed as ferricyanide units determined at pH 8·8 and at 20° with sodium pyrophosphate–HCl–EDTA buffer (see Appleby & Morton, 1959*a*). Extinction values were measured at the appropriate $\lambda_{max.}$ as shown. Protein was estimated according to Lowry *et al.* (1951) (see Methods section).

Stage	Fraction	Enzymic activity			Spectrophotometric measurements					
					U.v. band		Soret band			
		Units/mg. of protein	Total units	Recovery (%)‡	$\lambda_{max.}$ (mμ)	E	$\lambda_{max.}$ (mμ)	E	$\frac{E_{a.v.}}{E_{Soret}}$	$\frac{E_{a.v.}}{\text{Protein concn. (mg./ml.)}}$
	Original enzyme	5300	10 200	—	264	12·0	423·5	11·25	1·06	3·11
1	Ppt.	4800	7 500	73	268	4·46	423	7·50	0·59	1·72
	Supernatant	—	0	0	260	3·0	422	0·15	20·0	—
2	Recombined material*	4620	3 130	31	264	0·182	423	0·170	1·07	2·68
	(Supernatant	3025	955	9·5	261	0·230	423	0·152	1·51	3·65
	Nucleotide-depleted (Ppt.	1465	285	3·3	263	0·101	422	0·102	0·99	2·6
	material† Supernatant	1490	940	11·0	268	0·104	422	0·157	0·66	1·65

* Corresponds to 0·5 ml. of the original enzyme solution.
† Corresponds to 0·42 ml. of the original enzyme solution.
‡ These values refer to the original enzyme solution.

ratio $E_{261\,m\mu}$/protein concn. (mg./ml.) was 51. When the nucleotide-depleted enzyme so obtained was dialysed anaerobically at 0° against 0·05 M-sodium lactate–0·01 mM-EDTA, pH 6·8, for 5 hr., no crystals were obtained. A small amount of amorphous pink precipitate collected by centrifuging at 25 000 *g* for 20 min. at 0–4° contained only 18 % of the enzymic activity of the nucleotide-depleted enzyme; most of the activity was in the supernatant fraction.

Recombination of polynucleotide and flavohaemoprotein. Two separate portions, each of 0·5 ml., of once-crystallized cytochrome b_2 (3·8 mg./ml.) were dialysed against 0·7-saturated $(NH_4)_2SO_4$ containing 0·1 M-sodium lactate and 0·02 mM-EDTA at pH 5·9 and at 0°. After 4 hr., amorphous precipitates appeared in each dialysis sac.

The contents of one dialysis sac were centrifuged at 25 000 *g* for 25 min. at 0–4°, and the precipitate so obtained was dissolved in 0·6 ml. of 0·5 M-sodium lactate–0·1 mM-EDTA, pH 6·8, and at 0°. This solution of nucleotide-depleted enzyme, and the material in the unopened dialysis sac, were both dialysed against 0·05 M-sodium lactate–0·01 mM-EDTA, pH 6·8.

The turbidity of the contents of the unopened dialysis sac rapidly disappeared as $(NH_4)_2SO_4$ was removed. After about 2 hr., however, turbidity again appeared in this unopened sac but not in the other. Dialysis was continued overnight and then both sacs were opened.

The preparation in the unopened sac contained a heavy amorphous precipitate. However, a few crystals, having the appearance characteristic of recrystallized enzyme, were detected. Only slight turbidity appeared in the nucleotide-depleted preparation.

The results of the spectrophotometric and enzymic measurements of the various fractions are shown in Table 5.

Enzymic hydrolysis of the deoxyribose polynucleotide. Crystalline ribo- and deoxyribo-nucleases were dissolved in 0·5 M-sodium lactate–0·05 M-$MgCl_2$, pH 7·0, to give a concentration of 1 mg./ml. Approx. 0·05 ml. of ribonuclease was added to 0·5 ml. of a dilute solution of cytochrome b_2 in 0·5 M-sodium lactate–0·05 M-$MgCl_2$, pH 7·0, and 0·05 ml. of deoxyribonuclease was added to a second portion of the same cytochrome b_2 solution. The control preparation contained cytochrome b_2 and 0·05 ml. of 0·05 M-$MgCl_2$ only. The three preparations were dialysed against 0·05 M-sodium lactate–0·01 mM-EDTA, pH 6·8. After 14 hr. crystals appeared both in the control and in the preparation incubated with ribonuclease, but not in the preparation incubated with deoxyribonuclease. Whereas enzymic hydrolysis of the deoxyribopolynucleotide prevented crystallization by the standard procedure, it did not cause any appreciable change in the enzymic activity of the preparation (Table 6).

Studies of the incorporation of labelled phosphorus into the nucleotide component of cytochrome b_2

It appeared possible that some exchange or incorporation of inorganic phosphate into the polynucleotide component might occur during oxidation of lactate. This activity was therefore determined by measuring the amount of isotope (^{32}P) incorporated into cytochrome b_2 at pH 7·4 during oxidation of lactate in the presence of mM-sodium phosphate containing ^{32}P, as compared with similar tubes with lactate omitted. The cytochrome b_2 was precipitated at 0° with 50 % (v/v) of acetone, washed with 50 % (v/v) acetone at −15° and then dissolved in 0·01 N-NaOH for determination of radioactivity. Separate experiments were carried out with cytochrome b_2 itself, oxygen, cytochrome *c*, ferricyanide and methylene blue as electron acceptors. In further experiments, incorporation of ^{32}P into added adenosine diphosphate (to form adenosine triphosphate) was measured, essentially as described by Nielsen & Lehninger (1955). Methylene blue and cytochrome *c* were used as electron acceptors, with and without a dialysed extract of yeast added as a source of possible transferring

Table 6. *Effect of ribonuclease and deoxyribonuclease on the properties of crystalline cytochrome* b_2

Portions of cytochrome b_2 were incubated with crystalline pancreatic ribonuclease or deoxyribonuclease respectively as described in the text and were then dialysed anaerobically for 14 hr. against 0·05 M-sodium lactate–0·01 mM-EDTA, pH 6·8. Lactic dehydrogenase activities were determined at 20° and at pH 7·4 with 0·05 mM-heart-muscle cytochrome *c* or at pH 8·8 with ferricyanide as in Table 5 and are expressed in the units of Appleby & Morton (1959*a*).

	Enzymic activity						
	Total ferricyanide units			Total cytochrome *c* units			Ferricyanide units* / Cytochrome *c* units
Treatment	Ppt.	Supernatant	Total	Ppt.	Supernatant	Total	
Cytochrome b_2 only	2835	1500	4335	2340	1240	3580	1·21
With ribonuclease	2230	1464	3694	—	—	—	—
With deoxyribonuclease	0	3600	3600	0	2620	2620	1·37

* This ratio is given for the supernatant material.

† Corresponds to 0·42 ml. of the original enzyme solution.

‡ Corresponds to 0·5 ml. of the original enzyme solution.

‡‡ These values refer to the original enzyme solution.

enzymes and essential cofactors. However, in no case was there significant incorporation of ^{32}P, either into cytochrome b_2 or into adenosine diphosphate.

Nitrogen and sulphur content of cytochrome b_2

Nitrogen. With five replicates of approx. 1 mg. each, the total N of crystalline cytochrome b_2 was found to be $15{\cdot}1 \pm 0{\cdot}4$ %. It was estimated that the dried crystals contained 0·71 % of sodium lactate (see Appleby & Morton, 1959*b*). Hence the lactate-free enzyme contains 15·2 % of N. The minimum weight/mole is 80 000 g. (Appleby & Morton, 1959*b*), so that there is 0·065 % of haem N, 0·069 % of riboflavin N and approx. 0·9 % of nucleotide N (assuming 15 nucleotide residues/mole of enzyme-bound haem). Thus cytochrome b_2 contains approx. 14·2 % of amino acid N.

Sulphur. Dr K. W. Zimmerman (Microanalytical Laboratory, University of Melbourne) found that the crystalline enzyme contained 0·74 % of S.

DISCUSSION

Homogeneity of crystalline cytochrome b_2

The sedimentation and electrophoresis of the recrystallized enzyme was carried out to determine whether more than one protein was present.

Only one component was detected with the ultracentrifuge (Fig. 1) and, since there was little or no spreading of the boundary, it is unlikely that any protein other than cytochrome b_2 is present. Whereas nucleotide-free and intact cytochrome b_2 were separated by electrophoresis at pH 8·8 (as described above), no such separation was observed during sedimentation at pH 6·8.

The results obtained by electrophoresis are complicated by the dissociation of the polynucleotide component of the enzyme. As shown by Figs. 2 and 3, at pH 7·05 and pH 8·8 some free polynucleotide appeared as a fast-moving anion. At pH 6·65, 7·05 and 8·8 the haemoprotein peak remained symmetrical for a considerable period; the nucleotide-depleted protein fraction was separated only after electrophoresis for 269 min. at pH 8·8 (Fig. 3). The results in Fig. 5 and Table 1 establish that the leading anionic protein fraction, fraction N ($\lambda_{max.}$, 265 mμ; $E_{265\ m\mu}/E_{422\ m\mu}$ 0·96), is closely similar to the original enzyme ($\lambda_{max.}$, 266 mμ; $E_{266\ m\mu}/E_{423\ m\mu}$ 0·93) and thus contained intact cytochrome b_2. However, fractions B and D ($\lambda_{max.}$, 272 mμ; $E_{272\ m\mu}/E_{423\ m\mu}$ 0·67 and 0·54 respectively) clearly contained nucleotide-depleted enzyme. Since the nucleotide-depleted enzyme, having a lower net negative charge/mole, would migrate more slowly than the intact enzyme, this finding accounts for the asymmetry and the separation of the protein peak after prolonged electrophoresis (Figs. 2, 3).

Since the ratio of $E_{\text{Soret band}}$/protein concn. (mg./ml.) was almost constant for all fractions obtained at pH 8·8 (Table 1), the only protein material present was a haemoprotein with the absorption spectrum of cytochrome b_2. No flavoprotein or any other protein not containing protohaem was detected. The small amount of haemoprotein in fraction F (Table 1, and Figs. 4, 5) undoubtedly arose as a contamination during removal of fraction F from the electrophoresis cell. A trace of riboflavin phosphate, dissociated from the enzyme, was detected in fraction F by fluorescence and by paper chromatography, but no flavoprotein was present (Table 1).

Since the results revealed interaction between the enzyme and the buffer systems, which were of differing composition, the electrophoresis mobilities at different pH values could not be used to obtain the isoelectric point of the protein. The minimum solubility of cytochrome b_2 is at about pH 4·0.

The minimum weight/mole of crystalline cytochrome b_2 as estimated from iron, haem and flavin determinations is approx. 80 000 g. (Appleby & Morton, 1959*b*). This is a more reliable estimate than the earlier value (97 000 g.) reported by us (Appleby, 1957; see also Dixon & Webb, 1958). The amino acid analyses of the intact enzyme (Appleby, Morton & Simmonds, 1960; Morton, 1958) indicate a minimum weight (with one residue of histidine) of approx. 13 000, which is consistent with a value of 80 000 g./mole of enzyme-bound haem (see Morton, 1958). Nygaard (1958) has recently confirmed our earlier value (97 000 g.) but his preparation appears to be contaminated with ribonucleotides. The value of 230 000 g./mole of haem given by Boeri & Tosi (1956) is undoubtedly too high, probably due to impurities in the enzyme preparation (see Appleby & Morton, 1959*b*; Boeri & Cutolo, 1958).

From the nucleotide analyses (Table 3) the weight of polynucleotide/mole of enzyme-bound haem is approx. 5000 g. Thus the nucleotide-free enzyme has a minimum weight/mole of approx. 75 000 g. and the polynucleotide comprises about 6 % of the dry weight of the crystalline enzyme.

Deoxyribose polynucleotide of crystalline cytochrome b_2

The results presented here conclusively establish that the pronounced absorption of crystalline cytochrome b_2 near 265 mμ (Appleby & Morton, 1954, 1959*a*, *b*) is due to a deoxyribonucleotide associated with the crystalline enzyme. In enzyme recrystallized several times, both deoxyribose and thymine were identified by at least two procedures, whereas no ribose or uracil could be detected. Since much ribonucleotide is present before crystallization, but remains in the mother liquor (Table 4),

there is clearly a specific association of the protein of cytochrome b_2 and the deoxyribonucleotide, as reported previously (Morton, 1955, 1958; Appleby, 1957). Nygaard (1958) has since reported the preparation of yeast lactic dehydrogenase containing ribonucleotides. Table 4 shows that ribonucleotides are associated with partially purified preparations of cytochrome b_2 when prepared according to Appleby & Morton (1954, 1959*a*), but remain in the mother liquor on crystallization of the cytochrome b_2. Pellets of once-crystallized cytochrome b_2 collected from the mother liquor by centrifuging are sometimes overlain by a small amount of white amorphous precipitate composed mainly of ribonucleic acid. This contaminant may also cause an elevated value for the ratio $E_{\text{u.v. band}}/E_{\text{Soret band}}$ of once-crystallized enzyme (see Appleby & Morton, 1959*b*). However, no ribonucleotide has been detected in recrystallized enzyme and Nygaard's (1958) findings can probably be attributed to non-specific contaminants in his preparation of cytochrome b_2.

The base composition of the deoxyribonucleotide associated with crystalline cytochrome b_2 is given in Table 3. There are approximately equimolecular amounts of adenine and thymine, and of guanine and cytosine, consistent with the requirement of the theory of Watson & Crick (1953) for DNA. The quotient (moles of adenine + moles of thymine)/(moles of guanine + moles of cytosine) for the deoxyribonucleotide of cytochrome b_2 is 2·60 (from Tables 2 and 3) and that for the DNA of baker's yeast is 1·79 (Zamenhof & Chargaff, 1950). The ratio found for calf-thymus DNA is 1·20 (from Table 2) and is in reasonable agreement with the value of 1·36 reported for ox-thymus DNA by Chargaff & Lipshitz (1953). Hence the deoxyribonucleotide of cytochrome b_2 differs substantially from DNA of yeast and this supports the view that it is a specific component of cytochrome b_2.

Having regard to the difficulties of choosing appropriate standards there is reasonable agreement of analytical results based on the estimation of nucleotide phosphorus, sugars and of bases. The use of extinction coefficients based on ox-thymus DNA will cause some error in the estimation of nucleotide and of sugar, since the base composition differs substantially from that of the cytochrome b_2 polynucleotide (see above). Moreover, the amount of colour in the Ceriotti (1952) reaction for deoxyribose varies with the nature of the nucleotide. The phosphorus analyses are probably the most reliable and these indicate 14–15 nucleotide units/mole of enzyme haem.

The nucleotide units are undoubtedly polymerized, since the polynucleotide does not pass through a cellulose dialysis sac, and it is not eluted from paper under conditions which elute mononucleotides (see Results section). Moreover, the polynucleotide migrates as a single anionic component during electrophoresis (Fig. 3), and is depolymerized by deoxyribonuclease (see Results section and Table 6).

The polynucleotide is dissociated by high concentrations of ammonium sulphate (see Results section). Hence cytochrome b_2 prepared by salting-out procedures (Bach, Dixon & Zerfas, 1946; Boeri, Cutolo, Luzzati & Tosi, 1955) will contain little or no deoxyribopolynucleotide.

The slight excess of adenine over thymine (Table 3) may indicate that the crystalline enzyme contains an adenine-containing component, other than the deoxyribopolynucleotide. We have observed a pronounced absorption at about 340 mμ in the clear extract obtained by heating a solution of crystalline cytochrome b_2 in neutral lactate at 100° for 3 min. and dispersing the denatured protein in 0·7-saturated ammonium sulphate at 0–4°. This absorption peak was not detected in extracts obtained by heating with hydrochloric acid or perchloric acid at 80° for 1 hr. It is not due to either lumichrome or pyruvate and it is unlikely to be due to reduced di- (or tri-)phosphopyridine nucleotide, since Boeri & Tosi (1956) found less than 0·1 mole of nicotinic acid moiety/mole of haem in their preparation of yeast lactic dehydrogenase.

Function of the nucleotide

The results (Tables 5 and 6) show that the intact nucleotide is essential for crystallization of cytochrome b_2 as described by Appleby & Morton (1959*a*). After dissociation by salt, the nucleotide apparently does not recombine in the specific manner necessary for crystallization, although an amorphous precipitate is formed. However, as yet no other function has been found for the polynucleotide. It is not essential for dehydrogenase activity.

Cytochrome *b* of animal tissues appears to be essential for formation of adenosine triphosphate accompanying oxidation of reduced diphosphopyridine nucleotide (see Chance & Williams, 1956). Pinchot (1953, 1957) found that a polynucleotide component is essential for oxidative phosphorylation catalysed by intracellular particles from *Alcaligenes faecalis*. It therefore appeared possible that cytochrome b_2, having a polynucleotide as an integral part of the enzyme, might catalyse a phosphorylation accompanying lactate oxidation. However, no uptake or exchange of inorganic phosphate could be demonstrated with labelled phosphorus (see Results section).

The relationship between polynucleotides (or nucleic acids) and the synthesis of proteins is now well established [see, for example, Brachet (1957) and Chantrenne (1958)]. The incorporation of

amino acids into the protein of isolated nuclei from calf thymus is dependent on deoxyribonucleic acid (Allfrey, Mirsky & Osawa, 1957). It is possible therefore that the polynucleotide of cytochrome b_2 is a component associated with the synthesis of this haemoprotein.

Nucleotropomyosin, which contains a ribonucleotide, was purified by Hamoir (1951) by precipitation at neutral pH and at low ionic strength, as was cytochrome b_2. Unlike cytochrome b_2, however, tropomyosin also crystallizes by similar procedures in the absence of the nucleotide.

Cytochrome b_2 also resembles avidin, a non-crystalline protein which contains about 10% of a deoxyribopolynucleotide (Fraenkel-Conrat, Ward, Snell & Ducay, 1950). Intact avidin is insoluble in water but soluble in 0·2M-potassium phosphate; electrophoresis in potassium phosphate buffer gave a fast-moving anionic nucleotide component and a water-soluble nucleotide-free protein (Fraenkel-Conrat *et al.* 1950; Fraenkel-Conrat, Snell & Ducay, 1952).

SUMMARY

1. Sedimentation of crystalline cytochrome b_2 (lactic dehydrogenase) of baker's yeast in an ultracentrifuge revealed only one component, having a sedimentation coefficient ($S_{20,w}$) of 7·7s.

2. Electrophoresis at pH 6·65, 7·05 and 8·8 showed partial dissociation of a polynucleotide component of the enzyme. Separation of nucleotide-depleted enzyme from intact enzyme was detected after electrophoresis for 269 min. at pH 8·8. There was no separation of a flavoprotein from a haemoprotein. The results indicate that crystalline cytochrome b_2 contains only one major protein component.

3. There are approx. 15 (polymerized) deoxyribonucleotide residues/mole of haem in the crystalline enzyme. The base composition (adenine, 6; thymine, 5; guanine, 2; cytosine, 2 moles) of the polynucleotide differs considerably from that of yeast deoxyribonucleic acid. Recrystallized cytochrome b_2 contains no detectable ribose or uracil, indicating the absence of ribonucleotides.

4. Dissociation of the deoxyribopolynucleotide of cytochrome b_2 by 0·7-saturated ammonium sulphate or by digestion with pancreatic deoxyribonuclease prevented crystallization of the enzyme by anaerobic dialysis at low ionic strength. An amorphous precipitate was formed on recombination of the polynucleotide and nucleotide-depleted enzyme.

5. The nucleotide is not essential for dehydrogenase activity. No uptake or exchange of inorganic phosphate during oxidation of lactate catalysed by the crystalline enzyme could be demonstrated. The possible function of the polynucleotide is discussed.

6. There is approx. 0·56% of phosphorus, 0·74% of sulphur and 15·2% of nitrogen in the crystalline enzyme, and approx. 14·2% of nitrogen in the nucleotide-free enzyme.

7. The properties of cytochrome b_2, a crystalline deoxyribonucleoprotein, are compared with those of other nucleoproteins.

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REFERENCES

Allfrey, V. G., Mirsky, A. E. & Osawa, S. (1957). *J. gen. Physiol.* **40**, 451.

Appleby, C. A. (1957). Ph.D. Thesis: University of Melbourne.

Appleby, C. A. & Morton, R. K. (1954). *Nature, Lond.*, **173**, 749.

Appleby, C. A. & Morton, R. K. (1959*a*). *Biochem. J.* **71**, 492.

Appleby, C. A. & Morton, R. K. (1959*b*). *Biochem. J.* **73**, 539.

Appleby, C. A., Morton, R. K. & Simmonds, D. H. (1960). *Biochem. J.* **75**, 72.

Bach, S. J., Dixon, M. & Zerfas, L. G. (1946). *Biochem. J.* **40**, 229.

Beavan, G. H., Holiday, E. R. & Johnson, E. A. (1955). In *The Nucleic Acids*, vol. 1, p. 493. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press Inc.

Boeri, E. & Cutolo, E. (1958). *Abstr. Comm. 4th int. Congr. Biochem., Vienna*, no. 5–36, p. 60.

Boeri, E., Cutolo, E., Luzzati, M. & Tosi, L. (1955). *Arch. Biochem. Biophys.* **56**, 487.

Boeri, E. & Tosi, L. (1956). *Arch. Biochem. Biophys.* **60**, 463.

Brachet, J. (1957). *Biochemical Cytology.* New York: Academic Press Inc.

Ceriotti, G. (1952). *J. biol. Chem.* **198**, 297.

Chance, B. & Williams, G. R. (1956). *Advanc. Enzymol.* **17**, 65.

Chantrenne, H. (1958). *Annu. Rev. Biochem.* **27**, 35.

Chargaff, E. & Lipshitz, R. (1953). *J. Amer. chem. Soc.* **75**, 3658.

Chargaff, E., Vischer, E., Doniger, R., Green, C. & Misani, F. (1949). *J. biol. Chem.* **177**, 405.

Dixon, M. & Webb, E. C. (1958). *Enzymes*, p. 479. London: Longmans, Green and Co. Ltd.

Edward, J. T. & Waldron, D. M. (1952). *J. chem. Soc.* p. 3631.

Fraenkel-Conrat, H., Snell, N. S. & Ducay, E. D. (1952). *Arch. Biochem. Biophys.* **39**, 80.

Fraenkel-Conrat, H., Ward, W. H., Snell, N. S. & Ducay, E. D. (1950). *J. Amer. chem. Soc.* **72**, 3826.

Hamoir, G. (1951). *Biochem. J.* **48**, 146.
Hanes, C. S., Hird, F. J. R. & Isherwood, F. A. (1952). *Biochem. J.* **51**, 25.
Hurlbert, R. B., Schmitz, H., Brumm, A. F. & Potter, V. R. (1954). *J. biol. Chem.* **209**, 23.
King, E. J. (1951). *Quantitative Clinical Biochemistry*, 2nd ed. London: J. and A. Churchill Ltd.
Levene, P. A. & Mori, T. (1929). *J. biol. Chem.* **83**, 803.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
McNutt, W. S. (1954). *J. biol. Chem.* **210**, 511.
Markham, R. (1955). In *Modern Methods of Plant Analysis*, vol. 4, p. 246. Ed. by Paech, K. & Tracey, M. V. Berlin: Springer-Verlag.
Markham, R. & Smith, J. D. (1949). *Biochem. J.* **45**, 294.
Martin, E. M. & Morton, R. K. (1956). *Biochem. J.* **64**, 221.
Morton, R. K. (1954). *Biochem. J.* **57**, 595.
Morton, R. K. (1955). *Soc. biol. Chem., India: Silver Jubilee Souvenir*, p. 177.
Morton, R. K. (1958). *Rev. pure appl. Chem.* **8**, 161.
Nielsen, S. O. & Lehninger, A. L. (1955). *J. biol. Chem.* **215**, 555.
Nygaard, A. P. (1958). *Biochim. biophys. Acta*, **30**, 450.
Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
Pinchot, G. B. (1953). *J. biol. Chem.* **205**, 65.
Pinchot, G. B. (1957). *Biochim. biophys. Acta*, **23**, 660.
Tristram, G. R. (1953). In *The Proteins*, vol. 1, part 1, p. 181. Ed. by Neurath, H. & Bailey, K. New York: Academic Press Inc.
Watson, J. D. & Crick, F. H. C. (1953). *Nature, Lond.*, **171**, 737.
Weil-Malherbe, H. & Green, R. H. (1951). *Biochem. J.* **49**, 286.
Wyatt, G. R. (1955). In *The Nucleic Acids*, vol. 1, p. 243. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press Inc.
Zamenhof, S. & Chargaff, E. (1950). *J. biol. Chem.* **187**, 1.

Biochem. J. (1960) **75**, 269

The Stimulation of Transphosphorylation by Alkali-Metal Ions

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A number of enzyme reactions involving the polyphosphate chain of nucleoside triphosphates show an absolute requirement for, or a stimulation by, alkali-metal ions. This requirement is usually met by potassium ions, but sodium, rubidium and ammonium ions have been reported to serve in some cases. Examples include fructokinase (Lardy, Parks & Ben-Gershom, 1957), myosin adenosine triphosphatase (Kielley, Kalckar & Bradley, 1956; Bowen & Kerwin, 1954), pyruvate kinase (Kachmar & Boyer, 1953) and glutathione synthetase (Snoke, Yanari & Bloch, 1953). However, the majority of the enzyme-catalysed reactions which involve nucleoside triphosphates have not been tested for an alkali-metal requirement. Unless it is specifically tested for, such a requirement may easily be missed because many reagents, including adenosine triphosphate (ATP), are commonly used in the form of their sodium or potassium salts. It is likely therefore that many more reactions utilizing adenosine triphosphate possess a requirement for alkali-metal ions than are recognized to do so at present.

In a previous paper (Lowenstein, 1958) a non-enzymic transphosphorylation was described which involves the transfer of the terminal phosphate group of adenosine triphosphate to orthophosphate. The products of the reaction are inorganic pyrophosphate and adenosine diphosphate (ADP). The reaction shows an absolute dependence on bivalent-metal ions (M^{2+}):

Adenosine triphosphate + orthophosphate $\xrightarrow{M^{2+}}$ adenosine diphosphate + pyrophosphate. (1)

Of those tested, Ca^{2+}, Mn^{2+} and Cd^{2+} ions were the most effective.

The work presented here had two objects: the first was to provide a chemical basis for the alkali-metal requirement of enzyme-catalysed transphosphorylations. The second was to study variations in the amount of pyrophosphate formed in the non-enzymic reaction. These were observed to occur with reaction mixtures identical in every respect, except that different bases (such as potassium hydroxide, sodium hydroxide and diethanolamine) were used to neutralize the solutions of adenosine triphosphate employed in the experiments. A preliminary account of this work has been given (Lowenstein, 1959).

EXPERIMENTAL

Materials and reagents

Diethanolamine salt of adenosine triphosphate. The barium salt of adenosine triphosphate (ATP) was obtained from the Sigma Chemical Co., St. Louis, Mo., U.S.A., and from the Nutritional Biochemical Corp., Cleveland, Ohio,

U.S.A. Diethanolamine was obtained from the Matheson Co. Inc., Norwood, Ohio, U.S.A., and was purified by distillation under reduced pressure. Dowex-50 resin (8% cross-linked, 200–400 mesh, H^+ form) was obtained from The Dow Chemical Co., Midland, Mich., U.S.A. The resin was cycled through the sodium and acid forms, and was washed thoroughly with water before use.

Barium ATP 2 g. was suspended in 6 ml. of water in a 25 ml. beaker. A graduated centrifuge tube was filled with 14 ml. of a 50% (v/v) suspension of Dowex-50 resin (H^+ form) and the suspension was centrifuged. The volume of the resin was measured and adjusted to 7 ml. by the addition or subtraction of small amounts of resin and repeated centrifuging. When the desired volume of packed resin had been obtained the water was decanted. The following operations were performed on ice or in a cold room. The resin (7 ml.) was added slowly to the suspension of barium ATP with thorough stirring. The ATP dissolved very slowly at first, but, after a certain amount of resin had been added, a point was reached where the characteristic white slurry of barium ATP suddenly disappeared. This was usually accompanied by the conversion of the well-dispersed resin into a sticky mass. The remainder of the resin was added slowly with thorough stirring. After the addition of the resin was complete, stirring was continued for 5 min. The resin was filtered off on a sintered funnel and resuspended in 3 ml. of water. The suspension was stirred thoroughly and filtered on a sintered funnel. The washing of the resin was repeated twice more. The combined filtrates were neutralized to pH 3–5 by the addition of diethanolamine. This was most conveniently done by using the liquid base (m.p. 23°). A check of the total amount of diethanolamine added was made by weighing out an excess of the base and weighing the remainder at the end of the preparation. The pH was raised at this point to minimize the hydrolysis of ATP in the acid solution obtained from the resin treatment. Residual barium was then precipitated from the solution by the dropwise addition of 2N-H_2SO_4. If the solution became too cloudy to judge the completeness of precipitation it was centrifuged, and the addition of H_2SO_4 was continued until no further precipitate formed. This point is harder to judge than is the case in, say, the precipitation of barium from solutions of its chloride. It was best to add a drop of H_2SO_4 to the clear solution and to wait a few minutes to see whether the solution remained clear, before deciding that precipitation of barium was complete. The clear, barium-free solution was neutralized to pH 9·0 by the addition of diethanolamine, the pH being checked first on indicator papers, and near the desired pH on a meter. The concentration of ATP was determined spectrophotometrically ($\epsilon_{260\ m\mu}$ $15{\cdot}4 \times 10^3$), and was adjusted to the desired value, usually 0·1 M, with water. In a few preparations the concentration of ATP at the end of the preparation was less than 0·1 M. In these cases the cold solution of ATP was placed in a round-bottomed flask and some water was removed from the cold solution in a rotary drier. No appreciable breakdown of ATP occurred in the course of this treatment. The concentration of ATP was redetermined and adjusted to the desired value.

Other bases, such as 2-amino-2-hydroxymethylpropane-1:3-diol, can be used in place of diethanolamine in the above method, and a variety of metal-free base salts of ATP can thus be prepared.

Alkali-metal and ammonium salts. Rubidium chloride and CsCl were of a purified grade obtained from E. H. Sargent and Co., Chicago, Ill., U.S.A. Tetramethylammonium chloride was obtained from Eastman Organic Chemicals, Rochester, New York. Other salts were of reagent grade.

Radioactive phosphate. Carrier-free [^{32}P]orthophosphate was obtained from the Atomic Energy Commission, Oak Ridge, Tennessee, U.S.A. It was purified by paper chromatography with solvent system 2 (Lowenstein, 1957); the orthophosphate spot was detected by radioautography and was eluted with water.

Analytical methods

'Non-extractable phosphorus'. The analyses for [^{32}P]-orthophosphate incorporated into the reaction product were described by Lowenstein (1958). Caesium and tetra-alkylammonium salts were found to interfere with the extraction by causing the formation of a yellow precipitate. This tended to cling to the sides of the extraction tubes and to be dispersed in the 2-methylpropan-1-ol (*iso*butanol) layer, making its removal difficult. The extraction procedure was modified for samples containing caesium or tetra-alkylammonium salts. The reaction was stopped by placing the reaction mixture on a column of Dowex-50 (H^+ form, 200–400 mesh, 8% cross-linked) with a resin bed 1 cm. in diameter and 5 cm. high, and not by acidifying the reaction mixture with H_2SO_4. Immediately after the mixture had soaked into the resin the column was washed with 2 × 10 ml. of water. This treatment resulted in the removal of the interfering substances. Measured portions of the combined eluates were then assayed for the amount of [^{32}P]orthophosphate incorporated into the reaction product.

RESULTS

In order to facilitate comparisons, all reaction rates are expressed as the amount of pyrophosphate formed per millilitre of reaction mixture, irrespective of the actual volume used. All reaction rates quoted approximate closely to being initial rates since the reactions were stopped when they had proceeded 4% or less. The course of the reaction with time was demonstrated previously (Lowenstein, 1958).

Stimulation of transphosphorylation by potassium ions. The effect of 0·1 M-KCl on the rate of transphosphorylation is shown in Table 1. The results show that the stimulating effect can be considerable (100%), and that it is not an analytical artifact. An ATP/Mn^{2+} ion ratio of 1·11 was used in this experiment. The importance of the ATP/bivalent metal ratio in relation to the magnitude of the stimulation by alkali-metal ions is indicated later.

Effect of the concentration of potassium ions on transphosphorylation. The influence of the KCl concentration on the rate of transphosphorylation is shown in Fig. 1. At an ATP/Mn^{2+} ion ratio of 1·11, this rate is increased 28% in the presence of 0·01 M-KCl, doubled in the presence of 0·1 M-KCl, and trebled in the presence of 0·4 M-KCl. The possibility

of an inflexion of the curve at high KCl concentrations suggests that there might be more than one type of stimulating effect.

Specificity of univalent-metal-ion stimulation. The effect of 0·1 M-alkali-metal and ammonium salts is shown in Table 2. At the ATP/Mn^{2+} ion ratio chosen for this experiment (1·0) all substances tested show a stimulating effect on the reaction. The effect of KCl is, however, much greater than that of the other cations. Lithium chloride, NaCl, CsCl and NH_4Cl show a stimulating effect of between 24 and 29 %. Rubidium chloride forms an apparent exception to this group, and shows a stimulation of 41 %, but this is still considerably less than that of KCl, namely 73 %. A comparison of KCl with K_2SO_4 on an equivalent basis shows KCl to be somewhat more effective than K_2SO_4. This is possibly indicative of an ionic-strength effect.

Effect of tetramethylammonium chloride. The possibility that the univalent-metal-ion stimulation of transphosphorylation is solely an ionic-strength

Table 1. *Stimulation of transphosphorylation by potassium ions*

The complete reaction mixture contained: 50 µl. of 0·05 M-[^{32}P]orthophosphate ($6·76 \times 10^5$ counts/min.); 250 µl. of 0·1 M-diethanolamine ATP, pH 9·0; 50 µl. of 0·45 M-$MnCl_2$; 50 µl. of M-KCl. Final volume was 500 µl.; temp., 38°; time of incubation, 3 hr.

Reaction mixture	Pyrophosphate formed (µm-mole/ml.)
Complete	172
KCl omitted	87
KCl omitted but added 5 sec. before end of incubation	90
$MnCl_2$ omitted	0
Zero time	0

Table 2. *Stimulation of transphosphorylation by alkali-metal and ammonium ions*

The complete reaction mixture contained: 50 µl. of 0·05 M-[^{32}P]orthophosphate ($3·98 \times 10^5$ counts/min.); 250 µl. of 0·1 M-diethanolamine ATP, pH 9·0; 50 µl. of 0·45 M-$MnCl_2$, and, except for a control, 50 µl. of M-chloride or of 0·5 M-sulphate as indicated. Final volume was 500 µl.; temp., 38°; time of incubation, 3 hr. The amount of pyrophosphate formed in the control was 82·6 µm-moles/ml.

Alkali-metal or ammonium salt	Stimulation of transphosphorylation (%)
LiCl	24
NaCl	28
KCl	73
RbCl	41
CsCl	29
K_2SO_4	64
NH_4Cl	27
Tetramethylammonium chloride	0

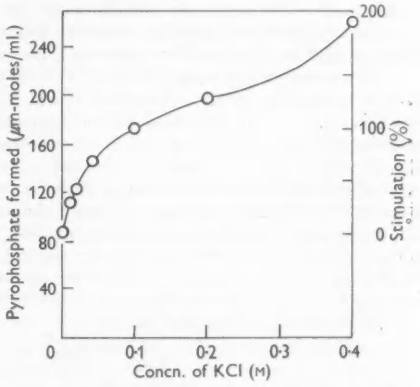


Fig. 1. Effect of potassium chloride concentration on transphosphorylation. The reaction mixture contained: 50 µl. of 0·05 M-[^{32}P]orthophosphate ($6·76 \times 10^5$ counts/min.); 250 µl. of 0·1 M-diethanolamine ATP, pH 9·0; 50 µl. of 0·45 M-$MnCl_2$; KCl at the concentrations indicated. Final volume was 500 µl.; temp., 38°; time of incubation, 3 hr.

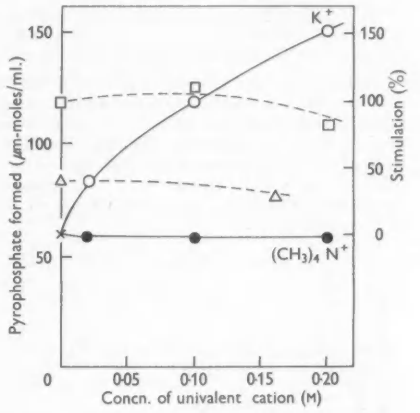


Fig. 2. Effect of tetramethylammonium chloride. The reaction mixture contained 50 µl. of 0·05 M-[^{32}P]orthophosphate ($1·0 \times 10^4$ counts/min.); 250 µl. of 0·1 M-diethanolamine ATP, pH 9·0; 50 µl. of 0·4 M-$MnCl_2$; KCl and tetramethylammonium chloride at the concentrations indicated. Final volume was 500 µl.; temp., 38°; time of incubation, 3 hr. ○, K^+ ion alone; ●, tetramethylammonium ion alone; △, tetramethylammonium ion as indicated plus 0·021 M-K^+ ion; □, tetramethylammonium ion as indicated plus 0·1 M-K^+ ion. All analyses in this experiment involved the Dowex-50-column step described in the Experimental section.

effect is ruled out by the experiment shown in Fig. 2. Tetramethylammonium chloride has virtually no effect on the alkali-metal-free reaction mixture over the concentration range 0–0·2 M. Potassium chloride shows the usual stimulating effect. The results obtained with mixtures of the two salts indicate that tetramethylammonium chloride may shift the effect of KCl slightly.

Alkali-metal stimulation in relation to the adenosine triphosphate/Mn^{2+} ion ratio. Fig. 3 demonstrates the effect of varying the concentration of Mn^{2+} ion in the absence of alkali-metal ions, and in the presence of KCl and LiCl. In each case the maximum rate of transphosphorylation is reached in a region where the ATP/Mn^{2+} ion ratio is 1 or less than 1, in agreement with earlier observations (Lowenstein, 1958). The alkali-metal stimulation is maximal in a region where the ATP/Mn^{2+} ion ratio is 1·1 in the presence of K^+ ion and 1·4 in the presence of Li^+ ion. This is demonstrated by replotting the data from Fig. 3 in the manner shown in Fig. 4. In the latter the control curve obtained with no added alkali metal has been subtracted from the rates obtained in the presence of KCl or LiCl. This method of plotting the results serves to make clear the quantitative differences between the stimulation obtained with K^+ and Li^+ ions.

The percentage stimulation of the alkali-metal ions, as distinct from the net stimulation, is maximal in a region where the ATP/Mn^{2+} ion ratio is about 2. This is demonstrated by replotting the data from Fig. 3 in the manner shown in Fig. 5. Broken lines are used at $MnCl_2$ concentrations of less than 30 μmoles/ml., because the relative errors in the measurement of the stimulation increase as the rate of transphosphorylation decreases. The percentage stimulation by KCl is still considerable when the ATP/Mn^{2+} ion ratio is 0·6, but stimulation by LiCl becomes zero at an ATP/Mn^{2+} ion ratio of 0·8. Figs. 3–5 therefore show the different effects of KCl and LiCl more clearly than does a comparison of these salts at a single ratio of ATP/Mn^{2+} ion.

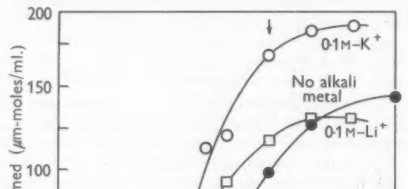


Fig. 3. Effect of K^+ and Li^+ ions in relation to the ratio ATP/Mn^{2+} ion. The reaction mixture contained: 50 μl. of 0·05 M-[^{32}P]orthophosphate ($3{\cdot}94 \times 10^5$ counts/min.); 250 μl. of 0·1 M-diethanolamine ATP, pH 9·0; $MnCl_2$ as indicated on the abscissa; where indicated, 50 μl. of M-KCl or M-LiCl. Final volume was 500 μl.; temp., 38°; time of incubation, 3 hr. The arrow indicates an ATP/Mn^{2+} ion ratio of 1·0.

Fig. 4. Effect of K^+ and Li^+ ions in relation to the ratio ATP/Mn^{2+} ion. Results of Fig. 3 have been replotted to show the net stimulation due to the addition of K^+ or Li^+ ions.

DISCUSSION

The results reported here demonstrate that alkali-metal ions stimulate the non-enzymic-transphosphorylation reaction from ATP to orthophosphate (equation 1). The most striking aspect of this stimulation is the observation that the effect of K^+ ions is exceptionally large compared with that

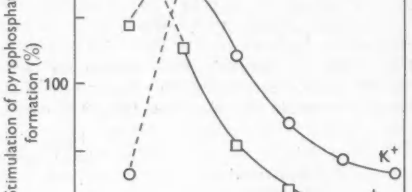


Fig. 5. Effect of K^+ and Li^+ ions in relation to the ratio ATP/Mn^{2+} ion. The results of Fig. 3 have been replotted to show the percentage stimulation due to the addition of K^+ or Li^+ ions.

of other alkali-metal ions (Table 2). The alkali-metal ions do not act simply by substituting for or satisfying the requirement for bivalent-metal ions. This is demonstrated in Table 1 and in Fig. 3, which show the bivalent-metal requirement to be absolute even in the presence of 0·1 M-alkali-metal ions.

In a previous paper (Lowenstein, 1958) the catalytic action of bivalent-metal ions was accounted for as follows: two of the negative charges of the polyphosphate chain of ATP are neutralized by chelation with the bivalent-metal ion. This greatly increases the probability of the attack on ATP by anions which would otherwise tend to be repelled. Chelation of the attacking agent to the ATP–bivalent-metal complex can further facilitate the phosphate-transfer reaction. The probability of successful attack on ATP by neutral nucleophilic agents is also increased, since chelation with the bivalent metal increases the electrophilic character of the phosphorus atom. The structures of a number of ATP–bivalent-metal complexes were discussed by Liebecq & Jacquemotte-Louis (1958) and by Lowenstein (1958).

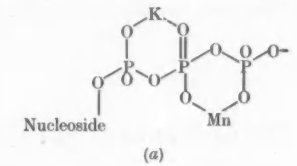
The simplest hypothesis to account for the effects described in this paper is that the alkali-metal ions exert their effect by neutralizing the negative charges on the polyphosphate chain of ATP which remain after the latter has combined with one bivalent-metal ion. According to this postulate, Li^+ ion would be expected to be the most effective ion because it possesses the smallest ionic radius of the univalent ions which were tested. Li^+ ion is indeed more effective than K^+ ion at high ATP/Mn^{2+} ion ratios (Figs. 3–5). But this simple postulate is inadequate to account for the different stimulating effects observed with different alkali metals at ATP/Mn^{2+} ion ratios in the region of 1 (Table 2; Figs. 3–5).

The available measurements of the stability constants of alkali metals with ATP provide no clue to the specificity of the stimulating effect of K^+ ions. Melchior (1954) found the stability constants for sodium ATP and potassium ATP to be the same (K, 10), although Alberty & Smith (1956) found slight differences (K_{K^+}, 11·5; K_{Na^+}, 14·3).

The observed effects may be accounted for in terms of the formation of complexes in which an alkali-metal ion as well as the bivalent-metal ion is chelated to ATP. A diagram of such a chelate is shown in Fig. 6*a*. It is proposed that the different stimulating effects reported here can be accounted for in terms of different modifications which each type of alkali-metal ion makes to the structure of the ATP–bivalent-metal chelate. The relative specificity of potassium may be because the charge–ionic radius relationship of the K^+ ion is such as to make its chelate with (ATP–bivalent metal)$^{2-}$ more active than that of the other alkali metals. Part of the observed effects must, however, be due to a simple neutralization of the remaining charges of ATP without chelation of the univalent ion, that is by ion-pair formation. This follows firstly because the NH_4^+ ion, which has no vacant co-ordination points available, also has a stimulating effect. Compared with potassium this amounts to about one-third under the experimental conditions used in Table 2. Secondly, the chelate shown in Fig. 6 (*a*) still possesses one negative charge. If this charge is also neutralized by a second alkali-metal ion this process would involve only ion-pair attraction, there being no possibility of the formation of five- or six-membered chelate rings with other oxygens of the polyphosphate chain. Fig. 3 shows that the much larger tetra-alkylammonium ion does not stimulate the reaction, and indicates that the ion-pair effect varies with the size of the cation. The magnitude of the ion-pair effect for different alkali-metal ions cannot, however, be estimated from the data presented here.

The ATP chelate shown in Fig. 6 (*a*) is not the only structure of its type which can be written. The possibility of position isomerism clearly exists. Thus the bivalent metal may also span the α- and β-phosphates of ATP, as was discussed previously (Lowenstein, 1958), and the position(s) occupied by the alkali-metal ion(s) would then also be different. Moreover, the chelate shown in Fig. 6 (*a*) does not have a planar structure. Fig. 6 (*b*) shows a possible conformation of this chelate. It consists of two chairs arranged so that the legs of one chair touch the back of the other chair. The two chairs do not face in the same direction: the top chair faces towards the reader and the bottom chair faces to his left.

Possible structures of complex ions containing one molecule of ATP and one metal ion were discussed by Melchoir (1954). It was suggested that


K
O
P
O
O
O

the structure of such complexes would vary with the size of the metal ion. Small metal ions would be completely surrounded by the polyphosphate chain of ATP, intermediate-sized metal ions would rest in a 'shallow cup' formed by the polyphosphate chain, and complexes with large metal ions would have an 'open' configuration. It was pointed out that such differences in the shape of the ATP molecule could profoundly affect the rate of enzyme-catalysed reactions. The non-enzymic reaction described here has an absolute requirement for a bivalent-metal ion, and shows a selective stimulation by K^+ and Rb^+ ions. This implies that in the alkali-metal-stimulated reaction the active complex contains at least two metal ions, and that its structure differs from those described by Melchior (1954). In close analogy, most or all enzyme-catalysed-transphosphorylation reactions from ATP show an absolute requirement for a bivalent metal. A number of such reactions show a selective stimulation by univalent cations, and here the active complex must also involve at least two metal ions. The structures proposed by Melchior (1954) are therefore also insufficient to account for the alkali-metal-ion stimulation of enzyme-catalysed transphosphorylations from ATP.

No complex-formation between the 6-amino group of adenine and the co-ordination points left vacant on the metal is indicated in Fig. 6. The balance of the evidence available at present (e.g. Spicer, 1952; Martell & Schwarzenbach, 1956) is against such an interaction. A similar interaction with the heterocyclic-ring system cannot be ruled out, but metals such as magnesium, calcium and potassium, which are involved in chelate formation with ATP in the cell, show a strong preference for chelating with oxygen as compared with nitrogen (Sidgwick, 1941).

SUMMARY

1. The stimulation by alkali-metal ions of a non-enzymic-phosphate transfer from adenosine triphosphate is described. The alkali-metal-ion stimulation does not abolish the absolute requirement by the reaction for bivalent-metal ions (M^{2+}).

2. Potassium ions produce a greater stimulation than other alkali-metal ions at adenosine triphosphate/M^{2+} ion ratios in the region of 1.

3. The observed effects are discussed in terms of ion-pair and of chelate formation.

4. The results provide a chemical basis for the alkali-metal-ion dependence or stimulation observed in many enzyme-catalysed-phosphate-transfer reactions involving adenosine triphosphate and similar substances.

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REFERENCES

Alberty, R. A. & Smith, R. M. (1956). *J. phys. Chem.* **60**, 180.
Bowen, W. J. & Kerwin, T. D. (1954). *J. biol. Chem.* **211**, 237.
Kachmar, J. F. & Boyer, P. D. (1953). *J. biol. Chem.* **200**, 669.
Kielley, W. W., Kalckar, H. M. & Bradley, L. B. (1956). *J. biol. Chem.* **219**, 95.
Lardy, H. A., Parks, R. E. & Ben-Gershom, E. (1957). *J. biol. Chem.* **227**, 231.
Liebecq, C. & Jacquemotte-Louis, M. (1958). *Bull. Soc. Chim. biol., Paris*, **40**, 67.
Lowenstein, J. M. (1957). *Biochem. J.* **65**, 197.
Lowenstein, J. M. (1958). *Biochem. J.* **70**, 222.
Lowenstein, J. M. (1959). *Fed. Proc.* **18**, 277.
Martell, E. A. & Schwarzenbach, G. (1956). *Helv. chim. acta*, **39**, 653.
Melchior, N. C. (1954). *J. biol. Chem.* **208**, 615.
Sidgwick, N. V. (1941). *J. chem. Soc.* p. 433.
Snoke, J. E., Yanari, S. & Bloch, K. (1953). *J. biol. Chem.* **201**, 573.
Spicer, S. S. (1952). *J. biol. Chem.* **199**, 301.

Biochem. J. (1960) **75**, 274

The Component Fatty Acids of *Sporidesmium bakeri* Lipids

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Under certain climatic conditions, such as warm autumn rains after summers of above-average temperatures, pastures in some New Zealand districts have been known to produce a virulent poison causing serious losses in sheep and cattle. Some of the affected animals show symptoms of photosensitization described as 'facial eczema', but the disease consists essentially in severe liver damage (Cunningham, Hopkirk & Filmer, 1942). Thornton & Percival (1959) have recently re-

ported that a high-sporing strain of *Sporidesmium* species [identified by the Commonwealth Mycological Institute, Kew, as *Sporidesmium bakeri* Syd. (Herb. I.M.I. no. 74473)] contains a hepatotoxin, which produces in both guinea pigs and lambs liver lesions similar to those encountered in 'facial eczema' disease. This discovery gave rise to an extensive study of this fungus, including the examination of its lipids. The fungus belongs to the class of Fungi imperfecti, order Moniliales, family Dematiaceae, the members of which are distinguished by the brown-and-black pigmentation of their mycelia and sometimes of their spores. The fatty acid composition of lipids from several members of the related Moniliaceae family such as *Aspergillus nidulans*, *Penicillium javanicum* and others, the mycelia and spores of which are hyaline or bright coloured, is already known (cf. Singh, Walker & Meara, 1955). In this paper we report the fatty acid composition of the lipids from *S. bakeri*, which, as far as we know, is the first member of the Dematiaceae to be thus examined.

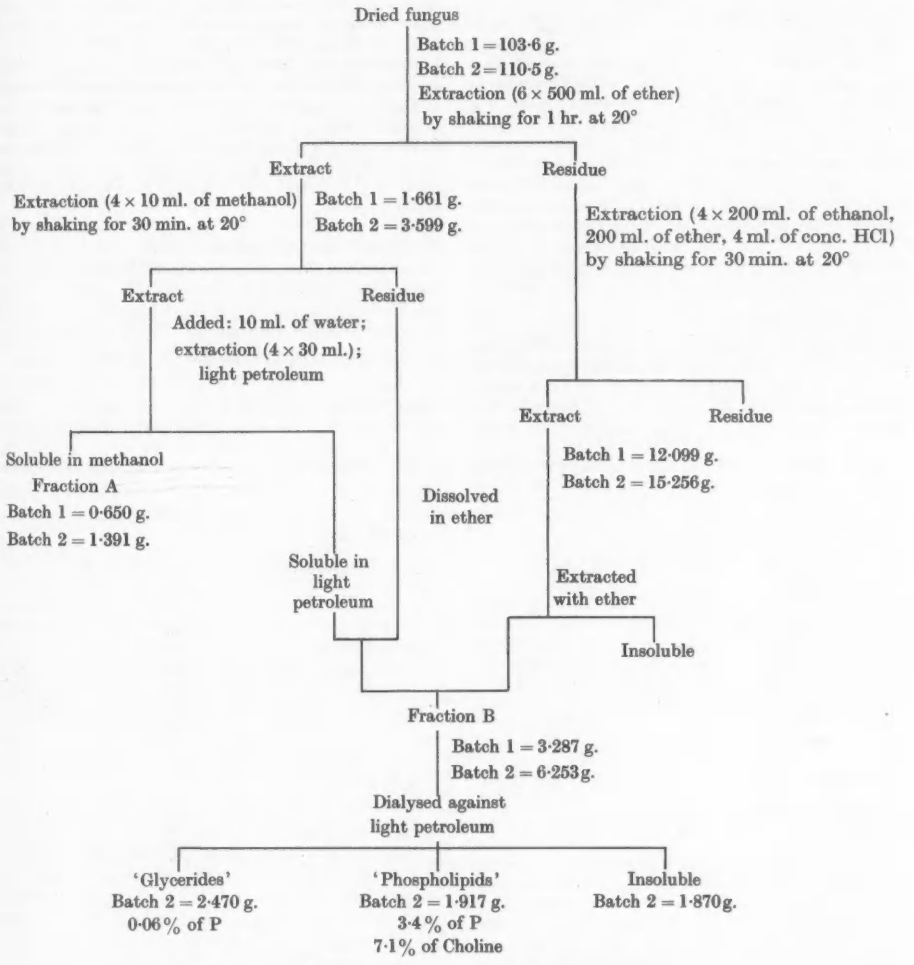

Fig. 1. Extraction scheme and weights of lipid fractions.

EXPERIMENTAL

Growth and collection of Sporidesmium bakeri

A high-sporing strain of *Sporidesmium bakeri* was surface-cultured on an infusion of 200 g. of potato and 200 g. of carrot/1000 ml. of water, in Roux bottles each containing 200 ml. of medium. After an incubation at 24° for 7 days, there was maximum spore development and the fungal mats were separated from the liquid culture medium by filtration through a Büchner filter. Two batches of 138 and 140 bottles of culture were obtained. In addition a lipid sample was examined, which represented a by-product obtained after the removal of the hepatotoxin from a large culture of *Sporidesmium* (700 Roux bottles).

Extraction and separation of the lipids

The common procedures used for the extraction and separation of the lipids from biological material were slightly modified to include partitioning of the lipid between aqueous 80% methanol and light petroleum, which was used by White (1958) for the concentration of hepatotoxic substances. The reason for applying this additional step was to establish whether the hepatotoxin has affinity to any specific fatty acids. The scheme employed in two batches of the fungus is outlined in Fig. 1. The only difference between the handling of the two batches was that the first was freeze-dried whereas the second was dehydrated by refluxing three times with acetone (1500 ml.) for 30 min. The third lipid sample corresponded to fraction B in Fig. 1.

Fraction B obtained from batch 2 contained an appreciable proportion of phospholipid (1·04% of P). This phospholipid was separated from the remaining lipids by dialysis against light petroleum (b.p. 40–60°) in a rubber membrane (van Beers, de Iongh & Boldingh, 1957). The yield of both components amounted to only 70·4% of the total material dialysed. This was due to the appearance during the dialysis of ethyl ether-insoluble material, which was discarded.

Analytical methods

The determination of fatty acids in the form of their methyl esters was carried out by gas–liquid chromatography. The various lipid fractions were saponified and after the removal of unsaponifiable matter the liberated fatty acids were converted into methyl esters as described by Hilditch (1956). The esters were separated, with a Pye Argon Chromatograph (Cambridge), the amount of each component being determined by measuring peak areas with a planimeter. The chromatographic columns (1·2 m. long, 4 mm. internal diameter) were packed with 45–85 mesh Celite 545 mixed with 20% (w/w) of Apiezon M (James & Martin, 1956) and operated at 200°, which made possible the separation of methyl oleate from methyl stearate. Another set of columns was packed with Celite, as above, impregnated with 20% of polydiethylene glycol succinate, which separated methyl linoleate from linolenate (Lipsky & Landowne, 1958). The use of these two liquid phases provided therefore means of determining the amounts of saturated and of mono-, di- and tri-ene esters in the C_{18} series.

Total phosphorus was determined colorimetrically according to the method of Allen (1940), and choline as the reineckate salt (Entenman, Taurog & Chaikoff, 1944).

RESULTS AND DISCUSSION

The weights of lipid fractions are shown in Table 1. The yield of lipids (4–6% of the dry weight of fungal mats) appears low when compared with that

Table 1. *Component fatty acids of three batches of* Sporidesmium bakeri *expressed as molar percentages*

		Batch 2				
			Fraction B			
Saturated acids	Batch 1. Fractions A + B	Fraction A	'Glycerides'	'Phospholipids'	Total	Batch 3. Fraction B
n-C_8	0·1	0·5	—	—	0·1	0·3
n-C_9	1·0	—	—	—	—	—
n-C_{10}	0·1	0·8	—	—	0·2	0·1
Branched-chain C_{11}	0·1	0·9	—	—	0·2	—
n-C_{11}	0·4	1·5	—	—	0·3	0·3
Branched-chain C_{12}	0·1	—	—	0·3	0·1	0·1
n-C_{12}	0·4	—	0·2	—	0·1	1·2
Branched-chain C_{13}	0·5	0·4	—	0·4	0·2	—
Branched-chain C_{14}	1·2	—	—	—	—	—
n-C_{14}	0·2	—	0·9	0·3	0·6	1·6
Branched-chain C_{15}	—	0·5	0·2	—	0·2	0·1
n-C_{15}	0·2	0·2	0·7	0·4	0·5	0·9
Branched-chain C_{16}	—	—	—	—	—	0·2
n-C_{16}	29·5	11·9	22·5	15·0	18·3	23·3
Branched-chain C_{17}	—	—	0·5	—	0·3	0·1
n-C_{17}	—	6·2	0·6	—	1·7	0·4
n-C_{18}	8·1	3·0	8·0	3·7	5·9	8·3
Unsaturated acids						
Palmitoleic	0·7	1·6	1·3	1·1	1·3	1·7
Oleic	15·0	6·7	9·9	5·7	8·2	11·3
Linoleic	41·3	62·1	52·5	71·0	59·0	46·5
Linolenic	1·1	3·7	2·7	2·1	2·8	3·6

of other fungi of the Moniliales order (Singh *et al.* 1955), but it is known that the lipid content of fungi is greatly influenced by the nature of the substrate (cf. Garrido & Walker, 1956).

The fatty acid composition of the three batches is shown in Table 1. The composition of batch 1 is given as a total (fraction A + fraction B) whereas the composition of the various fractions obtained from batch 2 (i.e. methanol-soluble lipid, glyceride and phospholipid) are given separately. The total composition of batch 2 has also been calculated on the basis of the weight of fatty acids obtained from each fraction. The composition of batch 3 (light petroleum-soluble lipid only) is given for comparison.

The fat moulds so far examined include *Aspergillus nidulans* (Singh & Walker, 1956), *Phycomyces blakesleeanus* (Bernhard & Albrecht, 1948), *Neurospora crassa* (Todd, Stone, Hechter & Nussbaum, 1957), *Penicillium* species (Hilditch & Meara, 1944), *P. javanicum* (Ward & Jamieson, 1934), *P. flavocinereum* (Singh, Shah & Walker, 1956*a*), *P. lilacinum* (Singh, Shah & Walker, 1956*b*) and *P. spinulosum* (Shimi, Singh & Walker, 1959). In general, the main fatty acid constituents (%, by wt.) are approximately as follows: palmitic (15–40), stearic (5–15), oleic (5–44), linoleic (13–43) and linolenic (trace–32). In *Phycomyces blakesleeanus*, however, there also occurs an isomer of linolenic acid, namely octadec-6:9:12-trienoic acid, tetracos-17-enoic acid and a hexacosenoic acid.

The present results show that the fatty acids from *Sporidesmium bakeri* Syd. lipids contain more linoleic acid (42–60%) than previously found in other moulds. Earlier work has shown that in general increased amounts of linoleic acid in moulds are associated with decreased amounts of palmitic acid. The fatty acids from *S. bakeri* Syd., however, display a high content of linoleic acid along with a considerable amount of palmitic acid (20–30%). The high linoleic acid content is accompanied by a reduction in the amount of oleic acid. The three batches of *Sporidesmium* show a similar pattern of fatty acid composition, but the 'phospholipid' fraction is notable for its high linoleic acid content (71%) compared with the 'glyceride' fraction, thereby resembling the relationships found between phospholipids and glycerides in liver fats.

The more sensitive gas–liquid-chromatographic technique, as compared with ester fractionation, has revealed the presence of small amounts of straight-chain fatty acids containing an odd number of carbon atoms in the molecule and branched-chain fatty acids (apparently of both *iso* and *anteiso* configuration), showing that fungi elaborate these constituents, as do animals and bacteria.

As mentioned above, the lipids in the fraction containing the hepatotoxin were examined separately to determine whether the fatty acids were selectively associated with the poison. The results show (batch 2, fraction A, Table 1) that the fatty acids in the hepatotoxin fraction comprise a similar range of fatty acids to that found in the other fractions. However, the amount of linoleic acid is higher and of palmitic acid and stearic acid is lower. This could be attributed to solubility factors.

SUMMARY

1. The component fatty acids of the lipids elaborated by *Sporidesmium bakeri* Syd., a fungus held responsible for the 'facial eczema' disease affecting sheep and cattle, have been determined by gas–liquid chromatography.

2. The main component acids have been shown to be linoleic acid (41–59%), palmitic acid (18–30%), oleic acid (8–15%) and stearic acid (6–8%) (molar percentages). In addition, minor amounts of linolenic acid, palmitoleic acid and of several straight-chain fatty acids containing an odd number of carbon atoms in the molecule and branched-chain fatty acids ranging from C_9 to C_{17} have been detected.

3. The fatty acid composition of *S. bakeri* lipids differs from that of other moulds inasmuch as an unusually high content of linoleic acid is accompanied by a substantial amount of palmitic acid.

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REFERENCES

Allen, R. J. L. (1940). *Biochem. J.* **34**, 858.
Bernhard, K. & Albrecht, H. (1948). *Helv. chim. acta*, **31**, 988.
Cunningham, I. J., Hopkirk, C. S. M. & Filmer, J. F. (1942). *N.Z. J. Sci. Tech.* A **24**, 185.
Entenman, C., Taurog, A. & Chaikoff, I. L. (1944). *J. biol. Chem.* **155**, 13.
Garrido, J. M. & Walker, T. K. (1956). *J. Sci. Fd Agric.* **7**, 233.
Hilditch, T. P. (1956). *The Chemical Constitution of Natural Fats*, 3rd ed., p. 572. London: Chapman and Hall Ltd.
Hilditch, T. P. & Meara, M. L. (1944). *J. Soc. chem. Ind., Lond.*, **63**, 112.
James, A. T. & Martin, A. J. P. (1956). *Biochem. J.* **63**, 144.
Lipsky, S. R. & Landowne, R. A. (1958). *Biochim. biophys. Acta*, **27**, 668.
Shimi, I. R., Singh, J. & Walker, T. K. (1959). *Biochem. J.* **72**, 184.
Singh, J., Shah, S. & Walker, T. K. (1956*a*). *J. sci. industr Res.* **15**c, 220.

Singh, J., Shah, S. & Walker, T. K. (1956*b*). *Biochem. J.* **61**, 222.
Singh, J. & Walker, T. K. (1956). *J. sci. industr. Res.* **15***c*, 222.
Singh, J., Walker, T. K. & Meara, M. L. (1955). *Biochem. J.* **61**, 85.
Thornton, R. H. & Percival, J. C. (1959). *Nature, Lond.*, **183**, 63.
Todd, D., Stone, D., Hechter, O. & Nussbaum, A. (1957). *J. biol. Chem.* **229**, 527.
van Beers, G. J., de Iongh, H. E. & Boldingh, J. (1957). *Int. Conf. Biochem. Probl. Lipids, Oxford*, p. 43.
Ward, G. E. & Jamieson, G. S. (1934). *J. Amer. chem. Soc.* **56**, 973.
White, E. P. (1958). *N.Z. J. agric. Res.* **1**, 433.

Biochem. J. (1960) **75**, 278

Constitutive Enzymes of the Developing Chick Embryo: Adenosine Deaminase

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The study of enzyme activities in developing tissues yields information only about the overall potential metabolic pattern and gives no clue to the nature of the chemical or biological stimulus which promotes the change in the differential rate of accumulation of a certain enzyme. If a specific stimulus to promote enzyme synthesis could be found, it would be possible to measure the capacity for enzyme synthesis of an embryonic tissue at any stage of its development. It is this capacity of embryonic cells to be controlled by their environment, at different stages of development, that may influence the cells' ultimate function. An attractive way of viewing the complex processes of differentiation is as 'a series of mutually dependent endogenously induced enzyme formations' (Rusch, 1954). The phenomenon of adaptive increase in enzyme activity is well known in bacteria (e.g. Monod, 1947; Spiegelman, 1948), as are increases in the activity of mammalian enzymes due to certain non-specific stimuli, such as the variation of dietary protein (e.g. Mandelstam & Yudkin, 1952), changes of altitude (Tappan, Reynafarje D., Potter & Hurtado, 1957), hormones or disease (Knox, Auerbach & Lin, 1956). However, relatively few cases of substrate-induced enzyme adaptations, other than that of tryptophan peroxidase (e.g. Knox, 1951), have been found in mammals.

If the development of the ability to produce certain enzymes during normal growth is due to a type of enzymic adaptation (otherwise known as enzyme induction) it might be expected that this capacity (with respect to any particular enzyme) could be prematurely stimulated by exposure of embryonic cells to an excess of substrate.

In the chick embryo only three cases of substrate-induced enzyme adaptation have so far been reported (Gordon & Roder, 1953; Gordon, 1956; Roeder, 1957). Arginase was found to be increased by 30–143% in the whole chick embryo during 5–14 days of incubation by injecting arginine into the egg (Roeder, 1957). Tryptophan peroxidase was increased tenfold in the developing chick embryo by injections of tryptophan during the third to ninth days of incubation (Gordon, 1956). Gordon & Roder (1953) found that adenosine-deaminase activity increased eightfold in whole chick embryos at 8 days of incubation after the eggs had been injected with adenosine 24 hr. earlier. These authors also found a sevenfold increase of enzyme activity in the liver at 14 days of incubation after seven consecutive daily injections of small amounts of adenosine.

No adaptive increase in lactic-dehydrogenase activities in the liver of the chick embryo was observed after injection of sodium pyruvate or lactate into the egg at various stages of development; similarly no adaptive increase in liver glutaminase was observed after injections of glutamine (J. B. Solomon, unpublished results). It was concluded that lactic dehydrogenase and glutaminase were constitutive enzymes, in that they are normally produced by the cell and are not susceptible to stimulation by the presence of their substrates. In view of the paucity of examples of enzyme induction in any type of embryo (Markert, 1958), we have attempted to confirm the results of Gordon & Roder (1953) on adenosine deaminase.

A preliminary report of these results has already been published (Palmer & Solomon, 1959).

EXPERIMENTAL

Tissues. Eggs from White Leghorn fowls (Appleby Farm Ltd., Ashford, Kent) were incubated at 37° in a Westernette incubator. The method of removal of whole chick embryos and yolk sacs from the eggs has previously been described (Solomon, 1958). Livers were obtained from White Leghorn chicks and cockerels which had been hatched from these eggs, except for some livers from 34-week-old Rhode-Island Red × Light Sussex cockerels.

Injections of adenosine. Adenosine (L. Light and Co. Ltd.) was recrystallized twice from hot water and made up as a sterile solution (16·7 mg./ml.) in 0·9% NaCl. A batch of eggs which had been incubated from 1 to 10 days was divided into a control group (about 15 eggs) and an experimental group (25 eggs). The latter group of eggs received sterile 0·9% NaCl containing 10, 20 or 25 mg. of adenosine, injected into the air sac by the technique of Gordon & Roder (1953). The control group was injected with the corresponding volumes of sterile 0·9% NaCl. The eggs were then sealed with paraffin wax and returned to the incubator for 18–22 hr. Tissues were removed from the experimental and control eggs by the method previously described (Solomon, 1958). Tissues from the control eggs were pooled for the control homogenate and tissues from all the experimental eggs were pooled for the one experimental homogenate.

Homogenates. Tissues were homogenized in 0·2 M-phosphate (Na_2HPO_4–NaH_2PO_4) buffer, pH 7·0, in a glass homogenizer with a Teflon pestle. Homogenates of whole chick embryos and yolk sacs were then centrifuged at 1000 *g* for 10 min. (Gordon & Roder, 1953) and the supernatant was poured off and assayed for adenosine-deaminase activity. Liver homogenates were not centrifuged except when they were to be used in a spectrophotometric method of estimation. Homogenate concentrations were, for whole embryo or yolk sac, 1 g. wet wt./5 ml., and, for liver, 1 g. wet wt./15 ml. of phosphate buffer.

Measurement of adenosine deaminase by Kalckar's method. The method of Kalckar (1944, 1947) as described by Gordon & Roder (1953) was used. This method is based on the decrease in absorption at 265 mμ due to the conversion of adenosine into inosine by adenosine deaminase. Adenosine (0·3 ml. of a 4 mg./ml. solution) in 0·2 M-phosphate buffer, pH 7·0, was added to 0·2 ml. of centrifuged homogenate and the mixture incubated at 38° for 30–60 min. Samples of centrifuged homogenate (0·2 ml.) from control embryos (four tubes) and experimental embryos (four tubes) together with the adenosine solution (0·3 ml.) were incubated at 38°. Similar samples but without adenosine were used as blanks. The reaction was stopped by the addition of 1·5 ml. of 0·5 M-perchloric acid, the mixture was centrifuged for 5 min. and a sample of the supernatant diluted 50 times with water. The absorption of the diluted supernatant at 265 mμ was corrected by subtracting that at 290 mμ from all solutions. No proportionality between concentration of homogenate and activity was found when centrifuged homogenates were assayed by this method. Activities when 0·2 ml. of homogenate was used were about one-twentieth of the value obtained by the ammonia method. A modification of Kalckar's method as used by Schneider & Hogeboom (1952) was then used. Two samples (0·05 ml.) of centrifuged homogenate (24 000 *g* for 30 min.) were each diluted to 3 ml. with 0·2 M-phosphate buffer, pH 7·0, and the diluted homogenates were poured into 1 cm. silica cells. Initial and final measurements were made in a spectrophotometer (Unicam SP. 500) at room temperature (20°). The extinction reading for the blank was set at 0·2 and at zero time adenosine (0·01 ml. of a 6 mg./ml. solution) was added to the other cuvette and mixed by stirring with a spatulate glass rod. Readings of E at 265 and 290 mμ were made against the blank. The two solutions were then quickly transferred to stoppered tubes and incubated at 38°, with shaking, for 1 hr. After cooling to 20° the extinctions were then read again in the same way. The difference between the corrected extinctions ($E_{265\ m\mu} - E_{290\ m\mu}$) before and after incubation gave the amount of adenosine deaminated.

Measurement of adenosine deaminase by the determination of ammonia. Samples (0·5 ml.) of homogenate were incubated with adenosine (0·15 ml. of a 4 mg./ml. solution) in 0·2 M-phosphate buffer, pH 7·0, for 30–60 min. The reaction was stopped by the addition of 15% (w/v) trichloroacetic acid solution (0·5 ml.). The ammonia was separated from the homogenate by the Conway (1947) microdiffusion technique and measured by a colorimetric method based on the phenol–hypochlorite reaction (Russell, 1944). Quadruplicate ammonia assays were made on the homogenate both with and without adenosine. The difference in amount of ammonia was then due to adenosine-deaminase activity. When the adenosine-deaminase activities of homogenates of embryos (about 15) receiving doses of 0·9% NaCl and embryos (about 25) receiving doses of adenosine were being compared, the one type of tissue (whole embryo, yolk sac or liver) of each group was pooled for one homogenate. The enzyme activity of each homogenate was then determined by quadruplicate assay as described above; the total number of determinations for two homogenates was 16. The coefficient of variation of the mean (amount of ammonia evolved) of any one group of quadruplicate assays was within the range ±0·7–9·0%. Activity was proportional to the amount of homogenate up to 0·6 ml. of homogenate of chick embryo or yolk sac.

Activity units. After subtraction of the amount of ammonia evolved by a homogenate without adenosine from the amount of ammonia evolved in the presence of adenosine the difference was multiplied by 15·7 to express the units of activity in terms of rate of breakdown of adenosine. One unit of activity of adenosine deaminase is defined as the amount required to deaminate 1 μg. of adenosine in 30 min. at 38°.

Protein determination. The method of Sutherland, Cori, Haynes & Olsen (1949) was used. Bovine-plasma albumin (The Armour Laboratories) dissolved in 0·1 M-phosphate buffer, pH 9·0, was used as a standard. Estimations were made within the range 10–120 μg. of albumin in 3·5 ml.

Statistical treatment. As the blank values in the ammonia method are about one-half of the values obtained in the presence of adenosine the t test has been used to test for significance of activity. Only values which are significant at the level P 0·05 have been used.

The significance of any increase in activity of the experimental homogenate above that of the control was assessed by calculating the standard error of the difference of the means (four determinations on each homogenate) and applying the t test. Only increases which are significant at the level P 0·05 are given in the tables.

RESULTS

The toxicity of adenosine to chick embryos varied considerably during development. It has been necessary to administer large doses of adenosine (25 mg.) to produce any significant increase of adenosine deaminase in the embryos, and the administration of this large dose has been possible only during the 6–8-day period of incubation. The large volume (1·5 ml.) of 0·9 % NaCl required to dissolve 25 mg. of adenosine practically filled the air sac; more concentrated solutions of adenosine started to crystallize at 37°. It was possible to inject eggs at 1–8 days of incubation with 20 mg. of adenosine in 1·2 ml. of 0·9 % NaCl without causing more than 30–50 % deaths. This is an extension of Gordon & Roder's work (1953) as these authors did not inject the embryos before the seventh day of incubation. The mean percentage survival figures are given in Table 1. The mean percentage survival figures for the controls ranged from 90 to 100 %.

Gordon & Roder (1953) obtained the greatest increase of adenosine-deaminase activity in the whole chick embryo by the injection of 25 mg. of adenosine into the egg at 7 days of incubation. Accordingly, we first tested for enzyme adaptation in eggs at this stage of development with the spectrophotometric method for adenosine deaminase used by Gordon & Roder (1953). No difference in activity between the experimental whole-embryo or liver homogenates and control homogenates was observed. However, the absolute units of activity were at most one-twentieth of those obtained by Gordon & Roder (1953) with the same method. When the modified spectrophotometric method due to Schneider & Hogeboom (1952) was used the values of adenosine-deaminase activity were of the same order as those obtained by the ammonia method (Table 2). To obtain good reproducibility by the modified spectrophotometric method it was necessary first to remove particulate material by high-speed centrifuging of the homogenates (24 000 *g* for 30 min.). This centrifuging should remove only up to 20 % of the total adenosine-deaminase activity of a homogenate as, when the conventional cell-fractionation technique of Schneider & Hogeboom (1950) was used with a liver of a 34-week-old cockerel, 80 % of the total activity remained in the supernatant fraction (Table 3). The activity of the enzyme in this fraction was optimum at pH 7·0.

Table 1. *Toxicity of single doses of adenosine at different stages of development of the chick embryo*

Incubation time when injected (days)	No. of embryos	Dose of adenosine (mg.)	Mean survival (%)
1–2	126	20	50
3–7	189	20	70
4–6	176	25	40
7–8	319	25	60
9–13	27	25	0

Adenosine-deaminase activity of untreated embryos

The adenosine deaminase/mg. of protein in the whole chick embryo remains constant at $7·7 \pm 0·6$ (23 results) activity units during 3–14 days of incubation (Fig. 1). The activity of homogenates of 2-day-old chick embryo at 1·0 unit is significantly lower (P 0·01) than that of embryos at later stages of development and it is probable that adenosine-deaminase activity has just appeared in the chick embryo at 2 days of incubation (24 hr. after the appearance of the primitive streak). The scattered values of adenosine-deaminase activity/mg. of protein in the developing yolk sac from 2 to 9 days of incubation have a mean value at $9·1 \pm 1·2$ (12 results) activity units. Liver-adenosine-deaminase activity has a mean value of $8·6 \pm 0·8$ (26 results) activity units/mg. of protein from 7 days of incubation to 34 weeks after hatching.

Table 2. *Comparison of adenosine-deaminase activities obtained by the ammonia and the modified spectrophotometric methods*

Homogenate	Incubation time when injected (days)	Dose of adenosine (mg.)	Adenosine-deaminase activity (units/mg. of protein)			
			Ammonia method		Spectrophotometric method	
			Control	Experimental	Control	Experimental
Yolk sac	1	20	2·8	3·4	6·0	7·8
Chick embryo	3	—	10·6	—	11·0	—
	4	20	14·5	16·2	16·7	16·4
	6	25	7·4	9·7	4·5	5·4
	7	25	—	—	10·6	14·2
	8	25	9·0	8·6	11·7	8·5
Livers: 9-day chick		—	8·6	—	5·6	—
Livers: 11-day chick		—	6·6	—	4·6	—
Livers: 38-week cockerel		—	12·0	—	12·5	—

Table 3. *Distribution of adenosine deaminase (ammonia method) among liver-cell fractions of a 34-week-old White Leghorn cockerel*

Cell fraction	Adenosine deaminase (% of total)	Adenosine-deaminase activity (units/mg. of protein)
Nuclei	8·3	6·3
Mitochondria	2·8	8·8
Microsomes	8·6	8·2
Supernatant	80·3	17·5

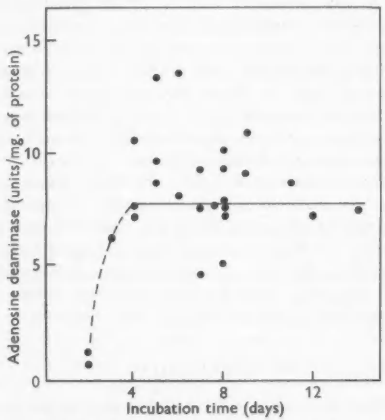

Fig. 1. Adenosine-deaminase activity/mg. of protein in centrifuged homogenates of whole chick embryos during early development.

Adenosine-deaminase activity of embryos dosed with adenosine

The injection of 20 mg. of adenosine into the air sacs of eggs incubated for 1–7 days produced, in two cases, an increase of enzymic activity (37 %) in the centrifuged homogenates of the whole embryos removed 24 hr. after injection (Table 4). With the embryos receiving 25 mg. of adenosine at 6, 7 and 8 days of incubation, four out of eight centrifuged homogenates at 24 hr. after injection showed 29–108 % increases of adenosine-deaminase activity (Table 4). However, increased activities were not obtained in the other experiments and the mean values of all the control and experimental adenosine-deaminase activities were not significantly different for either dose.

Adenosine deaminase was also measured in the yolk sacs of embryos dosed with 20 mg. of adenosine between 1 and 8 days of incubation. It was thought that the yolk sac, which is known to behave as a 'transitory liver' with respect to glycogen storage (Bernard, 1872) and to activities of glutamic, lactic and malic dehydrogenases (Solomon, 1957, 1958) during development, might be capable of increasing its adenosine-deaminase activity. However, five out of 11 adenosine-deaminase activities in the yolk sacs of the dosed embryos were decreased by 31–39 %, although the mean values of all the control and experimental activities were the same (Table 5).

Table 4. *Adenosine-deaminase activity of whole chick-embryo homogenates after injection of adenosine into the air sac*

Incubation time when injected (days)	Adenosine-deaminase activity (units/mg. of protein)		Percentage increase (P 0·05)
	Control	Experimental	
	Dose: 20 mg. of adenosine		
1	0·7	1·4	None
1	1·2	1·8	None
2	6·2	5·5	None
3	10·6	14·5	37
3	7·5	6·4	None
3	7·0	7·1	None
4	7·6	6·8	None
4	9·7	10·9	None
4	14·5	15·4	None
5	13·6	10·8	None
6	9·3	12·7	37
7	8·9	8·3	None
Mean	9·5	9·8	
	Dose: 25 mg. of adenosine		
6	4·5	7·6	69
6	7·4	9·7	None
6·5	7·5	9·1	None
7	10·2	21·0	108
7	7·1	9·2	29
7	7·5	14·8	97
7	8·9	9·4	None
8	9·0	8·6	None
Mean	7·7	11·2	

Table 5. *Effect of the injection of 20 mg. of adenosine on the adenosine deaminase of yolk sacs of chick embryos at different stages of development*

Incubation time when injected (days)	Adenosine-deaminase activity (units/mg. of protein)		Percentage decrease (P 0·05)
	Control	Experimental	
1	4·3	4·6	None
1	2·8	3·4	None
2	13·3	15·1	None
2	5·8	3·6	38
4	5·1	3·3	35
4	14·0	9·7	31
4	13·1	14·8	None
5	7·2	4·4	39
6	16·0	13·3	None
8*	25·4	16·2	36
8*	28·3	32·9	None
Mean	11·4	11·0	

* Dosed with 25 mg. of adenosine.

Although adenosine-deaminase activity does not appear to be localized in the liver, it was thought that the liver might be more likely to show enzyme adaptation than the whole chick embryo. As Gordon & Roder (1953) had obtained a sevenfold increase after seven injections of 10 mg. of adenosine during 7–13 days of incubation, it was thought that one dose of 20–25 mg. of adenosine might promote some increase of adenosine-deaminase activity. However, apart from an increase of 47 % in the liver of a 14-day-old embryo receiving 15 mg. of adenosine, no increased activity was produced with either single or multiple injections of adenosine (Table 6). Neither two injections of 15 mg. of adenosine at 24 hr. intervals nor five injections of 10 mg. of adenosine at 24 hr. intervals resulted in any increase of activity of the liver adenosine deaminase at 24 hr. after the last injection. As a short-lived adaptation might have occurred during the 24 hr. post-injection period an experiment was carried out on 80 eggs which had been incubated for 7 days. The eggs were injected with 25 mg. of adenosine and the livers removed at 6, 9, 12, 16, 18 and 24 hr. after injection. No significant increase of adenosine-deaminase activity occurred during this period. Chicks hatched from eggs dosed with 25 mg. of adenosine at 6 days of incubation showed no morphological abnormalities.

A preliminary report by Gordon (1952) on the effect of injecting adenosine into the chick egg suggested that liver-adenosine-deaminase activity could only be increased in embryos which were at least 20 days old. In view of the hypothesis (Burnet, 1956) that enzyme adaptation may be a preliminary step to antibody production and as it is known that the chick is not capable of producing antibodies until after hatching (Tyler, 1955), the reaction of the liver adenosine deaminase of chicks and fowls to injections of adenosine was briefly examined. In chicks and fowls such an experiment is severely limited not by the toxicity of adenosine but by the volume of solution of the poorly soluble adenosine that can safely be injected intraperitoneally. In this work the most that was injected daily into 20-week-old cockerels was 300 mg. of adenosine in 18 ml. of 0·9 % NaCl and four successive doses produced no ill-effects on the birds. A single dose of 300 mg. of adenosine injected into a 19-week-old cockerel produced a 37 % increase of liver-adenosine-deaminase activity after 24 hr.; four successive injections of 300 mg. of adenosine into a 20-week-old cockerel at 24 hr. intervals produced a 57 % increase in the adenosine deaminase of the liver, but not of the spleen (15·7 activity units/mg. of protein), at 24 hr. after the last injection (Table 6). However, the mean values for all the control and experimental chick embryo and chick livers were not significantly different.

The amount of ammonia evolved from homogenates of untreated embryos without substrate in all these determinations showed no significant variation during development. Both whole chick embryo and liver homogenates evolved $0{\cdot}18 \pm 0{\cdot}03$ (50 results) μg. of ammonia/30 min./mg. of protein, and yolk-sac homogenates evolved $0{\cdot}09 \pm 0{\cdot}02$ (10 results) μg. of ammonia/30 min./mg. of protein.

DISCUSSION

The distribution of adenosine deaminase in the various tissues of the chick has not been extensively studied. Whereas Conway & Cooke (1939) found that there was about ten times more adenosine-deaminase activity in rabbit spleen than in the liver, in this work it was found that in a 19-week-old cockerel the adenosine-deaminase activity/mg. of protein in the liver does not significantly differ from that in the spleen. Gustafson & Hasselberg (1951) have found that no variation of adenosine-deaminase activity/mg. of nitrogen occurred

Table 6. *Effect of injections of adenosine on the adenosine-deaminase activity of the livers of chick embryos, chick and fowls*

	Age when first injected (days)	Dose of adenosine (mg.)	Adenosine-deaminase activity (units/mg. of protein)		Percentage increase (P 0·05)
			Control	Experimental	
Embryos	6	2 × 15	9·1	9·8	None
	6	5 × 10	17·2	10·7	None
	7	15	7·2	7·7	None
	7	25	6·1	6·3	None
	7	25	7·8	7·2	None
	9	15	6·2	7·5	None
	14	15	6·3	9·3	47
Chicks and fowls	8	3 × 25	9·0	8·6	None
	133	300	7·3	10·1	37
	140	4 × 300	10·8	16·8	57
			Mean 8·7	9·4	

during the early development of *Paracentrotus lividus*. However, adenosine deaminase has been found to increase in mouse liver after injection of xanthine (Feigelson, Feigelson & Wood, 1954).

Contrary to earlier beliefs that embryonic differentiation was accompanied by the synthesis of many enzymes, it appears from recent work that many enzyme activities show only relative changes of activity during development (e.g. Shen, 1955; Moog, 1946, 1947, 1950; Solomon, 1957, 1958, 1959). It is unlikely that a tissue having high enzyme activity will respond to the stimulus of a large amount of substrate as there will always be sufficient enzyme to deal with even large amounts of substrate. This is one reason for testing enzymes known to have only low activities (e.g. adenosine deaminase and glutaminase). Even then the study of any such adaptation is severely limited, as adaptation may occur only at substrate doses very near the 50% lethal level, as was found for arginase in the chick embryo (Roeder, 1957). Gordon (1956) also reports that a major difficulty of studying enzyme adaptation is the high death rate of chick embryos injected with the various substrates. In this work adenosine has also been found to be toxic to the chick embryo and this may be partly due to its specific action *in vitro* of inhibiting cells from entering prophase (Hughes, 1952); no morphological or growth-inhibiting effects of adenosine have been noticed in these experiments.

No substrate-induced increase of adenosine deaminase at any stage of development of the chick embryo has been found in this work. This is contrary to the results obtained by Gordon & Roder (1953) after the injection of 25 mg. of adenosine in the whole chick embryo at 7 days of development and in the embryonic liver after seven successive doses of 10 mg. of adenosine. Moreover, it has been shown in this work that the activity of adenosine deaminase/mg. of protein does not show any significant increase in untreated whole chick embryos (after 2 days of incubation), yolk sacs or livers throughout development. This, again, is in disagreement with the results of Gordon & Roder (1953) on whole chick-embryo homogenates over a narrower range of development. These authors found that adenosine-deaminase activity/mg. of protein increased from about 2 units at 7 days of incubation to about 20 units at 14 days of incubation in untreated whole embryos. The range of activities obtained in the present work during this period of incubation was 4·5–14·9 units, but there was no upward trend in the values as time of incubation increased. From the results of the present experiments adenosine deaminase can be considered to be a constitutive enzyme throughout the development of the chick embryo.

SUMMARY

1. Adenosine-deaminase activity/mg. of protein does not vary significantly in homogenates of the whole chick embryo after 2 days of incubation or of its yolk sac from 2 to 14 days of incubation or in the liver from 7 days of incubation to 34 weeks after hatching.

2. Adenosine was injected into eggs at from 1 to 8 days of incubation but no significant increase of adenosine-deaminase activity in homogenates of the whole chick embryo or yolk sac was observed at 24 hr. after each injection.

3. Five successive doses of 10 mg. of adenosine injected at 24 hr. intervals into 6-day-old embryos produced no increased adenosine-deaminase activity in the livers of the embryos removed 24 hr. after the last dose.

4. Liver adenosine deaminase was not significantly increased by single or multiple injections of adenosine into embryos during 6–8 days of incubation or by similar injections into chicks or fowls.

5. Adenosine-deaminase activity was chiefly localized in the supernatant fraction of a liver homogenate of a 34-week-old cockerel and its optimum activity in this fraction was at pH 7·0.

6. Adenosine-deaminase activity/mg. of protein was similar in embryonic and cockerel liver, whole chick embryos and yolk sacs.

7. It is concluded from these results that adenosine deaminase is a constitutive and not an adaptive enzyme throughout the development of the chick.

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REFERENCES

Bernard, C. (1872). *C.R. Acad. Sci., Paris*, **75**, 55.
Burnet, F. M. (1956). *Enzyme, Antigen and Virus*. Cambridge University Press.
Conway, E. J. (1947). *Microdiffusion Analysis and Volumetric Error*. London: Crosby Lockwood and Son Ltd.
Conway, E. J. & Cooke, R. (1939). *Biochem. J.* **33**, 479.
Feigelson, P., Feigelson, M. & Wood, T. R. (1954). *Science*, **120**, 502.
Gordon, M. W. (1952). *Fed. Proc.* **11**, 220.
Gordon, M. W. (1956). *Progr. Neurobiol.* **1**, 83.
Gordon, M. W. & Roder, M. (1953). *J. biol. Chem.* **200**, 859.
Gustafson, T. & Hasselberg, I. (1951). *Exp. Cell Res.* **2**, 642.
Hughes, A. (1952). *Exp. Cell Res.* **3**, 108.

Kalckar, H. M. (1944). *Science*, **99**, 131.
Kalckar, H. M. (1947). *J. biol. Chem.* **167**, 445.
Knox, W. E. (1951). *Brit. J. exp. Path.* **32**, 462.
Knox, W. E., Auerbach, V. H. & Lin, E. C. C. (1956). *Physiol. Rev.* **36**, 164.
Mandelstam, J. & Yudkin, J. (1952). *Biochem. J.* **51**, 681.
Markert, C. L. (1958). In *Chemical Basis of Development*, p. 3. Ed. by McElroy, W. D. & Glass, B. Baltimore: Johns Hopkins Press.
Monod, J. (1947). *Growth*, **11**, 223.
Moog, F. (1946). *J. cell. comp. Physiol.* **28**, 197.
Moog, F. (1947). *J. exp. Zool.* **105**, 209.
Moog, F. (1950). *J. exp. Zool.* **115**, 109.
Palmer, D. & Solomon, J. B. (1959). *Biochem. J.* **72**, 28P.
Roeder, M. (1957). *J. cell. comp. Physiol.* **50**, 241.
Rusch, H. P. (1954). *Cancer Res.* **14**, 407.
Russell, J. A. (1944). *J. biol. Chem.* **156**, 457.
Schneider, W. C. & Hogeboom, G. H. (1950). *J. biol. Chem.* **183**, 123.
Schneider, W. C. & Hogeboom, G. H. (1952). *J. biol. Chem.* **195**, 161.
Shen, S. C. (1955). *Biological Specificity and Growth*, p. 73. Princeton: University Press.
Solomon, J. B. (1957). *Biochem. J.* **66**, 264.
Solomon, J. B. (1958). *Biochem. J.* **70**, 529.
Solomon, J. B. (1959). *Developmental Biol.* **1**, 182.
Spiegelman, S. (1948). *Symp. Soc. exp. Biol.* **2**, 286.
Sutherland, E. W., Cori, C. F., Haynes, R. & Olsen, W. S. (1949). *J. biol. Chem.* **180**, 825.
Tappan, D.V., Reynafarje D., B., Potter, V. R. & Hurtado, A. (1957). *Amer. J. Physiol.* **190**, 93.
Tyler, A. (1955). *Analysis of Development*, p. 520. Philadelphia: W. B. Saunders Co. Ltd.

Biochem. J. (1960) **75**, 284

Aromatic *N*-Deacylation by Chick-Kidney Mitochondria

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During an investigation of the metabolism of some *para*-substituted anilines it became desirable to convert the excreted *N*-acetyl compounds into their arylamines by enzymic rather than by acid hydrolysis, so reducing the risk of altering other parts of the metabolite molecules. Kynurenine formamidase of mammalian liver (Mehler & Knox, 1950) and of *Neurospora* (Jakoby, 1954), the only enzymes of this type described, appeared not to be suitable because of their high specificity for *o*-formamido compounds. There was, however, evidence that an enzyme more appropriate to our requirements was to be found in chick kidney. Shaffer & Bieter (1950) reported that after giving sulphanilamides to chicks much less of the N^4-acetyl conjugates could be found in kidney than in other tissues or blood. They also found that administered N^4-acetylsulphanilamides were excreted in the urine largely as the free bases; this was in contrast with mammalian species. Finally, these workers showed that chick-kidney homogenates deacetylated acetylsulphanilamides at a rapid rate.

Some of the properties of the chick-kidney enzyme have been explored and are now reported. In spite of the superficial similarity of the reaction it catalyses, the enzyme is quite distinct from kynurenine formamidase.

EXPERIMENTAL

Materials

Substrates. A few of the compounds tested as substrates were of commercial origin. In most cases the arylamines, obtained commercially, were acylated with the appropriate acid anhydride or acid chloride. Formylation was carried out with acetic formic anhydride (Dalgliesh, 1952). In every case the compound was recrystallized from a suitable solvent (e.g. aqueous ethanol) until all detectable arylamine had been removed. The *p*-acetamido derivatives of 3-phenoxypropionic acid, 4-phenoxybutyric acid and 5-phenoxyvaleric acid were kindly provided by Dr A. G. Caldwell. Compounds in the benzamido series were generously prepared for the present purpose by Dr J. H. Gorvin.

Buffers. Buffered solutions at a concentration of 0·1 or 0·2M were prepared in glass-distilled water. 2-Amino-2-hydroxymethylpropane-1:3-diol (tris) and 2-amino-2-methylpropane-1:3-diol (ammediol) were obtained from L. Light and Co. Ltd. The pH values of all solutions were measured at the concentration at which they were used, with a glass electrode assembly (W. G. Pye and Co. Ltd.) and temperature corrections were applied where necessary.

Other reagents. Most reagents were of A.R. grade and were used as such; others were purified, when necessary, by some standard procedure appropriate to the particular compound. *p*-Chloromercuribenzoic acid was a gift from Dr L. A. Stocken; 2:3-dimercaptopropanol was bought from Boots Pure Drug Co. Ltd. and was not purified. Tween 20 (Honeywill–Atlas Ltd.) and Triton X-100 (Rohm

and Haas Co., Philadelphia, Pa., U.S.A., and bought through Charles Lenning and Co. Ltd., London) were also used as received.

Enzyme preparations

Cross-bred fowls of both sexes and of different ages were killed by air embolism or by cervical section. Kidneys were quickly removed and either used directly (after chilling) or stored at −15°. Activity remained reasonably high during storage; over a period of 3 months there was a loss of only 15%.

Fresh or thawed tissue was homogenized in about 10 vol. of chilled medium in a loose-fitting, all-glass homogenizer of the type described by Potter & Elvehjem (1936) and held in an ice bath. The medium used was 0·25 M-sucrose containing mM-tris and mM-disodium ethylenediaminetetra-acetate (EDTA); it had a pH of 7·1. After centrifuging for 10 min. at 700 *g* the supernatant was centrifuged for a further 10 min. at 15 000 *g* in the no. 69402 angle head (previously chilled to −3°) of the MSE Magnum centrifuge with high-speed attachment (Measuring and Scientific Equipment Ltd.). After removal as completely as possible of the supernatant together with a shallow layer of loosely packed particles, the residue was transferred to the homogenizer and resuspended in the same volume of the sucrose–tris–EDTA medium as had been used originally. For experiments on the effects of metal ions EDTA was omitted throughout. Such suspensions, particularly in the presence of EDTA, were fairly stable at −15° but were not usually kept for more than 5 days. Suspensions prepared in buffered-electrolyte solutions lost activity much more rapidly on storage.

Rat liver was used as a source of kynurenine formamidase. Adult animals of the Laboratory Wistar strain were killed by cervical section; the livers were removed at once, chilled and homogenized in 5–10 vol. of 0·05 M-phosphate buffer, pH 7·4. Homogenates were centrifuged as described above and the supernatant from the second centrifuging was used in most experiments without further treatment. Such preparations were very stable at −15°. For comparative experiments on the effects of metal ions enzyme partially purified by the method outlined by Mehler & Knox (1950) was used.

Measurement of activity

Conditions were varied to suit experimental requirements. Typically, reactions were carried out in 4 in. × ½ in. test tubes held in a water bath at 38° and containing 1 μmole of substrate in 0·5 ml. of 0·1 or 0·2 M-phosphate buffer, pH 7·2–7·3, and were started by the addition of 0·5 ml. of chick-kidney suspension diluted in the sucrose–tris–EDTA medium, or of rat-liver preparation diluted in water. After 60 min. the reaction was stopped by the addition of either 4 ml. of 0·37 N-perchloric acid or of 2 ml. of 0·11 M-zinc acetate followed by 2 ml. of 0·09 N-NaOH; the contents of the tubes were thoroughly mixed and precipitates removed by centrifuging. The amount of liberated arylamine was determined in the supernatant solutions. Results were corrected for a small amount of non-enzymic hydrolysis that occurred with the more labile substances. A small correction for tissue blank had also to be applied when determinations were made in u.v. light.

Analytical methods

Arylamines. Products of enzymic deacylation were measured by several different spectrophotometric methods. A Unicam model SP. 500 instrument with a light path of 1 cm. was used throughout. (*a*) To amine in 2 ml. of a perchloric acid supernatant were added successively 1 ml. of 2 N-HCl, 0·5 ml. of 0·1% $NaNO_2$ solution and 0·5 ml. of 0·5% ammonium sulphamate solution, with intervals of at least 5 min. between the second and third additions. The diazonium compound was coupled with 1-naphthol by adding 1 ml. of a freshly prepared solution containing 1 vol. of 12% (w/v) 1-naphthol in ethanol and 10 vol. of 3 N-NaOH. Intensity of the colour, which developed immediately and was stable for at least 2 hr., was measured at 490 mμ. This method was suitable for determination of *ortho*- and *para*-substituted anilines (except *o*-aminophenol, *p*-phenylenediamine and *p*-dimethylaminoaniline) of the naphthylamines and of 4-aminoantipyrin. (*b*) For the determination of *meta*-substituted anilines diazotization was performed as in (*a*) and colour developed by adding 1 ml. of an aqueous 0·3% solution of *N*-(1-naphthyl)ethylenediamine dihydrochloride (Bratton & Marshall, 1939); intensity was measured at 550 mμ. (*c*) A method of wide applicability was to form the Schiff base of the arylamine with *p*-dimethylaminobenzaldehyde at pH 2·0–2·2 (Venkataraman, Venkataraman & Lewis, 1948). A sample of the supernatant after treatment with zinc acetate and NaOH was brought to 4·5 ml. with water and treated with 0·5 ml. of 0·5% solution of the reagent in acetic acid. Extinctions were measured at 445 mμ after about 30 min.; they remained constant for some hours. In later experiments it was found possible to increase sensitivity twofold and to save much time by adding 3 ml. of a modified reagent directly to the 1 ml. of reaction mixture. The modified reagent was made freshly each day by dissolving 240 mg. of *p*-dimethylaminobenzaldehyde in 12 ml. of acetic acid and diluting with 30 ml. of redistilled ethanol and 50 ml. of water. Even relatively large amounts of suspension, containing up to 100 μg. of protein, did not interfere with colour production and gave by themselves extinctions less than 0·02. (*d*) For the measurement of deacylation of two of the substrates none of the above methods was suitable, and in these instances determinations were based upon differences in u.v. absorption of acid solutions. Samples of the perchloric acid supernatants were suitably diluted in 0·1 N-HCl and readings were made at 240 mμ for the conversion of *p*-aminoacetanilide (ε 13 400) into phenylenediamine (ε 210) and at 242 mμ for the conversion of *p*-dimethylaminoacetanilide (ε 15 100) into *p*-dimethylaminoaniline (ε 1120). In a few of the experiments with this substrate the deacylation of *p*-acetamidoanisole (*p*-acetaniside; ε 11 000) to *p*-anisidine (ε 150) was determined in the same way at 245 mμ.

For each method separate calibrations were made with purified samples of the arylamines concerned. With several substrates it was possible to make comparisons of the different methods and these were in excellent agreement.

Protein. The method of Lowry, Rosebrough, Farr & Randall (1951) was used for determination of protein and was standardized with crystalline bovine serum albumin (The Armour Laboratories, Eastbourne). With samples of the particulate suspension it was found

advisable for quantitative recovery of protein to allow treatment with the alkaline copper reagent to continue for 2 hr.

RESULTS

Intracellular localization of activity

Homogenates of chick kidney, after centrifuging at 700 *g*, regularly showed a high order of activity ranging from 170 to 320 μmoles of *p*-acetamidoanisole hydrolysed/g. of tissue/hr. When such suspensions were centrifuged at 15 000 *g* only traces of activity could be measured in the supernatant; at least 80 % of the original activity, however, could be recovered in the resuspended residue. A second sedimentation and resuspension of the particles was accompanied by a slight decrease in total activity, but again only about 1 % of this could be found in the supernatant. Essentially the same results were obtained, though with a rather greater loss of total activity, when the particles were suspended in various concentrations of phosphate buffer at pH 7·3.

Several treatments were used in unsuccessful attempts to release the enzyme from its particulate association. After each treatment the suspension was centrifuged at 15 000 *g* and activity sought in the supernatant; recovery of total activity was determined either with a sample taken before centrifuging or from activity of the sedimented residue after resuspension in the sucrose medium. Although, as has been found by other workers with liver mitochondria, surface-active agents brought about a release of large amounts of protein, there was no associated release of activity. An outline of the results of these experiments is given in Table 1. Repeated freezing and thawing was also unsuccessful. In all further work suspensions of the sedimented particles were used.

Table 1. *Effect of some surface-active agents on protein distribution and total deacylating activity*

Portions of chick-kidney particulate suspension (5–6 mg. of protein/ml.) were treated at 5° and pH 7·2 for 20 min. Activity values are given as percentages of those found in untreated suspensions; 'protein released' is the percentage of total protein found in the 15 000 *g* supernatant after treatment. Activities of the supernatants, which never exceeded 2–3 % of the total, are not given.

Treatment	Protein released (%)	Total activity (%)
Control suspension	9	100
Cetyltrimethylammonium bromide (0·1 %)	31	3
Tween 20 (0·1 %)	51	105
Sodium deoxycholate (0·12 %)	60	73
Triton X-100 (0·12 %)	70	96
Digitonin (1 %)	94	13

Effect of time and enzyme concentration on rate of reaction

With a suitable amount of enzyme the deacylation of mM substrate was linear with time up to about 50 % of completion (Fig. 1). During a fixed incubation period a similar proportionality between deacylation and enzyme concentration (Fig. 2) was found for all substrates tested in this respect. For

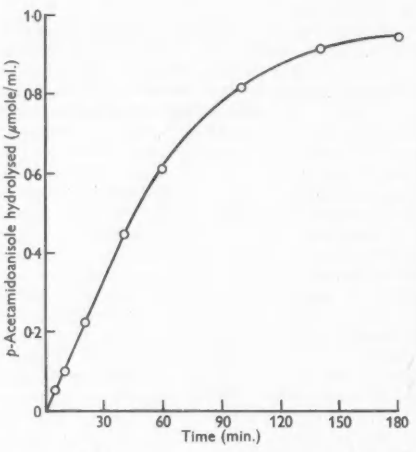

Fig. 1. Effect of time on course of reaction. *p*-Acetamidoanisole (10 μmoles) in 10 ml. of 0·1 M-phosphate buffer, pH 7·2, was incubated with enzyme (230 μg. of protein) at 38°. Enzyme was added after temperature equilibration and then samples (0·5 ml.) were removed at intervals into perchloric acid and analysed for *p*-anisidine.

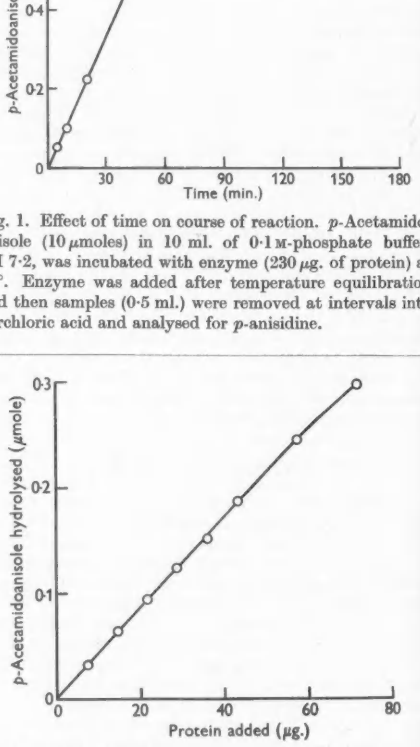

Fig. 2. Effect of varying enzyme concentration. Increasing amounts of chick-kidney suspension were incubated for 60 min. at 38° with 1 μmole of *p*-acetamidoanisole in 1 ml. of 0·1 M-phosphate buffer, pH 7·2.

the accurate determination of rates the enzyme concentration was adjusted so as to allow not more than 20 % of complete hydrolysis/hr.

Influence of pH *and of different buffers*

In 0·05 M-phosphate buffer a well-defined peak of activity was found at pH 7·2–7·3; the curve relating activity to pH was continued into the more alkaline range by 0·05 M-$NaHCO_3$–Na_2CO_3 buffer (Fig. 3). With no other buffer system could a clear optimum of activity be established, although in glycine buffer (Fig. 3) there was indication of an approaching peak towards the acid end of the useful range of this buffer. Pyrophosphate buffer produced a broad range of high activity extending from pH 7·6 to 8·4, with a slow decline on either side. In the presence of tris–HCl, tris–acetic acid, ammediol–HCl and triethanolamine–HCl buffers activity rose sharply from the acid end of the ranges to reach a plateau of activity between pH 8·2 and 9·2. On these plateaux rates were much the same in each of the four buffers and approximately equal to activity at the optimum found in phosphate buffer. Diethylbarbiturate (Fig. 3) and borate buffers were inhibitory; in each, activity was greatest at the highest pH tested (pH 9·1 and 9·8 respectively) and was not more than 40 % of that found in phosphate buffer at pH 7·3.

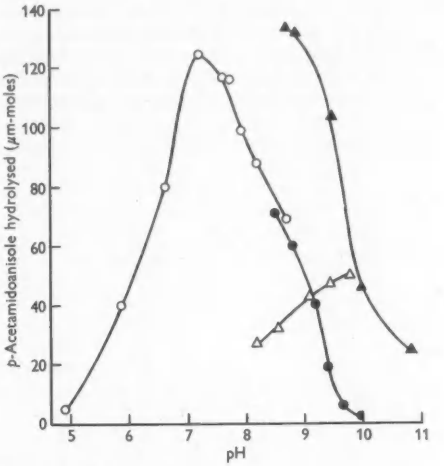

Fig. 3. Effect of pH and of buffer ion. *p*-Acetamidoanisole (1 μmole) was incubated with suspension (9 μg. of protein) in 1 ml. of 0·05 M-KH_2PO_4–Na_2HPO_4 (○), $NaHCO_3$–Na_2CO_3 (●), glycine–NaOH (▲) or sodium diethylbarbiturate–HCl (△) buffer for 60 min. at 38°.

Influence of temperature

The initial rate of deacylation of *p*-acetamidoanisole in 0·1 M-phosphate buffer, pH 7·2, was measured during 15 min. at temperatures between 14° and 50°. An optimum temperature of near 44° was found for this incubation period (Fig. 4). From an Arrhenius plot of the rates in the range 19–40° the energy of activation was calculated to be 15 300 cal. Above 40° heat inactivation of the enzyme was increasingly apparent.

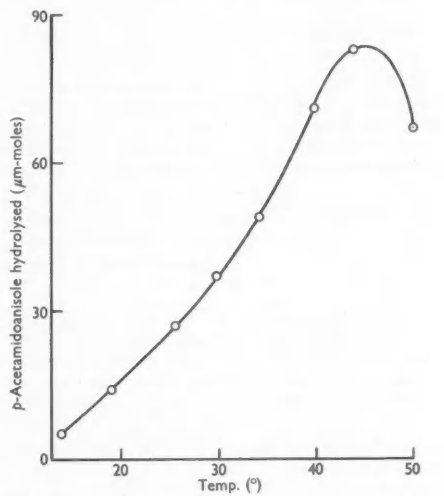

Fig. 4. Influence of temperature on the deacetylation of *p*-acetamidoanisole. Tubes containing 1 μmole of substrate in 1 ml. of 0·1 M-phosphate buffer, pH 7·3, were incubated for 7 min. at the indicated temperatures. The reaction was started by adding 0·05 ml. of suspension and stopped after 15 min. with 4 ml. of perchloric acid.

Inhibitors

A variety of compounds was tested for possible effects on the rate of deacylation of *p*-acetamidoanisole. Acetate (adjusted to pH 7·3) was without effect at 10 mM and inhibited only to the extent of 18 % at 100 mM. Monochloroacetate caused a 12 % reduction in rate at 5 mM and a 62 % reduction at 50 mM. Monoiodoacetate was rather more toxic; it reduced the rate by 17 % at 2 mM and by 50 % at 10 mM.

The enzyme (prepared in the absence of EDTA) was sensitive to inhibition by *p*-chloromercuribenzoate (Fig. 5); at 10 μM this compound caused about 30 % inhibition and at 100 μM complete inhibition. Three compounds containing thiol groups were tested for their ability to reverse this inhibition, but in every condition tested only partial reactivation could be demonstrated. Portions of

chick-kidney suspension, with and without inhibitor at 50 μM, were incubated in 0·25 M-sucrose containing 0·05 M-phosphate buffer, pH 7·2, at 38°. After 15 min. samples were diluted and added to tubes containing buffered substrate and different concentrations of the thiol compounds; the mean rate of hydrolysis during an incubation period of 1 hr. was then determined. In these conditions samples pre-incubated in the presence of inhibitor had 40–50 % of the activity of portions pre-incubated in its absence. Reduced glutathione at 2·5 mM consistently reversed inhibition to the greatest extent observed, but activity, as determined during the fixed time interval employed, never reached more than 80 % of the control level. Cysteine at the same or at higher concentrations was slightly less effective than glutathione. At the highest concentrations tested (25 mM) both compounds caused a slight, variable (5–15 %) stimulation of activity in control samples. At no concentration tested (0·25–25·0 mM) did 2:3-dimercaptopropanol effect any reversal of the inhibition produced by *p*-chloromercuribenzoate; at the higher concentrations it was itself slightly inhibitory.

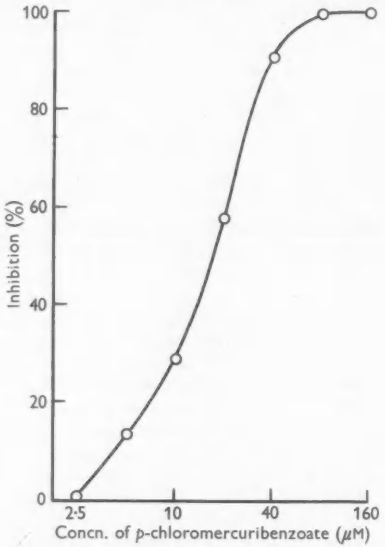

Fig. 5. Inhibition by *p*-chloromercuribenzoate of the deacetylation of *p*-acetamidoanisole. Substrate (1 μmole) and chick-kidney suspension prepared without EDTA (12 μg. of protein) were incubated with the indicated concentrations of inhibitor at 38° in 1 ml. of 0·1 M-phosphate buffer, pH 7·2.

Table 2. *Inhibition by metals of the deacetylation of p-acetamidoanisole by suspensions of chick-kidney particles*

Incubations were for 60 min. in 1 ml. of tris–acetic acid buffer containing 1 μmole of substrate and the indicated concentrations of inhibitor. Results are the mean values of at least two experiments; control tubes without inhibitor were included in each series. Enzyme was prepared without EDTA.

	Inhibition (%)			
Compound	Concn. 0·2 μM	Concn. 2·0 μM	Concn. 20·0 μM	Concn. 200·0 μM
$HgCl_2$	22	76	100	—
$CuCl_2$	9	78	95	—
$CdCl_2$	9	76	98	—
Zinc acetate	10	66	100	—
$AgNO_3$	0	42	100	—
$Pb(NO_3)_2$	—	14	69	83
$Ni(NO_3)_2$	—	11	—	80

From Table 2 it can be seen that the enzyme was very sensitive to inhibition by metal ions. In the presence of 2 μM-Hg^{2+}, -Cu^{2+}, -Cd^{2+} or -Zn^{2+} ions rates of deacylation were reduced to 20–35 % of control values. Even at 0·2 μM these metals caused distinct inhibition, most marked for Hg^{2+} ions. A slightly less-pronounced effect was produced by Ag^{+} ions and Pb^{2+} and Ni^{2+} ions were roughly one-tenth as inhibitory.

Arsenite produced a 62 % inhibition at 2 mM; at the same concentration cyanide was without effect.

Particle suspensions prepared on separate occasions in the absence of EDTA exhibited variable activities when these were determined also in the absence of EDTA. On the other hand, when the chelating agent was added (at 0·5 mM) to the assay tubes rates in the less-active preparations were usually increased; the largest stimulation seen in these conditions was approximately twofold.

Substrate specificity

Ability of the chick-kidney particles to deacylate a range of monosubstituted acylanilides was explored. Initial reaction rates were measured as already described; enzyme concentrations were necessarily varied over a wide range but, as before, values were calculated from amounts which resulted in the release of less than 0·2 μmole of arylamine from 1 μmole of substrate/hr. Within each experiment measurements of the rate of deacylation of a reference substrate (usually *p*-acetamidoanisole) were always included. Determinations of relative rate made in this way with different suspensions and at different times agreed well.

With several substrates initial rates were measured over a wide range of substrate concentration. Apparent K_m values were calculated from double reciprocal plots of the data (Lineweaver & Burk, 1934) fitted by the method of least squares.

Effects of ring substitution. The effect of introducing a *para* substituent into acetanilide was

generally to increase the relative rate of deacylation (Table 3). Only two substrates in this series, *p*-acetamidoaniline and *p*-acetamidoarsonic acid, were caused to react at a substantially slower rate. Determination of the apparent K_m values of some such substrates (Table 4) indicated that the increased rate at 1 mM concentration after *para* substitution was due at least in part to an increased affinity for the enzyme. From these values it can be seen that the relative rates of deacylation of other *para*-substituted compounds at 1 mM concentration is a rough indication of their affinities.

Ortho substitution had an opposite effect and was invariably followed by a profound fall in the rate of deacylation; *p*-acetamidobenzoic acid, for instance, was deacylated about 1000 times as rapidly at mM concentration as was the *ortho* compound. Rates of deacylation of these compounds (Table 5) were sometimes so slow as to prevent a determination of apparent K_m values. For *o*-acetamidoanisole the apparent K_m was found to be 10·2 mM (max. rate 0·72 μmole/mg. of protein/hr.); for *o*-acetamidochlorobenzene it was 3·4 mM (max. rate 1·3 μmoles/mg. of protein/hr.).

Substitution in the *meta* position had a tendency to reduce the rate of reaction (Table 5), but its effects were very much weaker than those of the corresponding *ortho* substitution. Only one substituent in this position, chlorine, effectively increased the rate over that of the unsubstituted compound.

Influence of the N*-acyl group.* With the six substrates tested in this respect the rates of deacylation of chloroacetyl derivatives were much faster than those of the corresponding acetyl compounds (Table 3). With acetanilide, for instance, the rate of deacylation was increased about eightfold by the introduction of chlorine. In the chloroacetyl series, however, the effect of *para* substitution was much less marked than in the acetyl series (Table 3); in fact the *p*-chloroacetyl derivatives of compounds containing a carboxylic group were split at about half the rate of chloroacetanilide. The apparent K_m values of four of these compounds were determined and are given in Table 4.

A similar large increase in the relative rate of deacylation of *ortho*- and *meta*-substituted compounds after *N*-chloroacetylation was also observed. For example *o*-chloroacetamidobenzoic acid was deacylated about 25 times as rapidly as *o*-acetamidobenzoic acid; it had an apparent K_m of 0·43 mM but its maximal rate of reaction was still only 2·1 μmoles/mg. of protein/hr.

Upon *N*-dichloroacetylation the relative rates fell to values very little higher than those of the *N*-acetyl derivatives.

Formylation was accompanied in every case tested by a marked fall in the relative reaction rate. With formanilide the relative rate was decreased to 8% and with *p*-formamidoanisole to 5% of

Table 3. *Influence of* para *substitution on the rate of deacylation of acetanilide and chloroacetanilide*

Substrates (1 mM) were incubated with enzyme in 0·1 M-phosphate buffer, pH 7·3, for 60 min. at 38°. Enzyme concentrations were adjusted to allow not more than 20% of substrate to be changed. Liberated arylamines were measured by methods described in the Experimental section. Each value is the mean of two or more determinations. Rates given are all relative to that of acetanilide.

Substituent	Relative rate of deacylation	
	N-Acetyl	*N*-Chloroacetyl
$AsO(OH)_2$	10	—
NH_2	60	—
CN	90	—
H	100	840
OH	115	—
$CO{\cdot}CH_3$	145	1300
$CO{\cdot}NH{\cdot}CH_2{\cdot}CO_2H$	150	925
NO_2	170	—
CHO	180	—
$O{\cdot}CH_2{\cdot}CH_2OH$	200	—
$O{\cdot}CH_3$	220	2530
CO_2H	220	500
$SO_2{\cdot}NH_2$	220	—
$N(CH_3)_2$	260	—
$O{\cdot}[CH_2]_{2-4}{\cdot}CO_2H$	280	—
$O{\cdot}CH_2{\cdot}CH_3$	310	—
CH_3	330	—
$CH_2{\cdot}CO_2H$	330	515
Cl*	380	—

* Compared, because of its low solubility, at a concentration of 0·5 mM.

Table 4. *Apparent K_m values of some* para*-substituted acylanilides*

Determinations were made as outlined in Table 3 with substrate concentration systematically varied in the range 0·05–5·0 mM. Maximal rates are given as μmoles/mg. of protein/hr.; K_m values are mM concentrations.

Substituent	*N*-Formyl		*N*-Acetyl		*N*-Chloroacetyl	
	K_m	Max. rate	K_m	Max. rate	K_m	Max. rate
H	7·4	5·6	0·91	10	0·14	32
$O{\cdot}CH_3$	1·2	16	0·59	18	0·61	122
CO_2H	1·3	17	0·23	5·4	1·0	15
$CH_2{\cdot}CO_2H$	2·6	19	0·078	18	0·12	38

those found with the acetamido compounds; with *p*-aminobenzoic acid, *p*-aminophenylacetic acid and *p*-aminohippuric acid the difference was less marked and the formyl derivatives were deacylated at 75, 20 and 40 % respectively of the rate of the corresponding acetyl compound. From Table 4 it can be seen that the slower deacylation of formamido compounds at mM concentration is probably a reflexion of the relatively high apparent K_m values of these compounds.

The effect of increasing the length of the *N*-acyl chain was also investigated. Deacylation, on the basis of relative rate, was most rapid with the *p*-propionamido derivatives of benzene, anisole and phenetole, and with the *p*-acetamido derivatives of benzoic acid, phenylacetic acid and hippuric acid. The relationship between relative rate and chain length is exemplified for these two groups in Fig. 6.

In Table 6 are listed the apparent K_m values of some longer-chain derivatives of *p*-aminobenzoic acid and *p*-aminophenylacetic acid. With both compounds there was an unexpected fall in apparent K_m in passing from the *N*-butyryl to the *N*-valeryl derivative; determinations made on separate occasions confirmed this finding. On further increasing the length of the *N*-acyl chain on *p*-aminobenzoic acid (Table 6) the apparent K_m value increased only slightly.

N-Succinyl derivatives of several of the compounds already mentioned were tested, but none was deacylated at a measurable rate. The effect of branched chains was not investigated.

N-Benzoyl derivatives. Low solubilities of benzamido compounds limited the investigation of substrates of this type, and only derivatives of *p*-aminobenzoic acid were tested. *p*-Benzamidobenzoic acid was hydrolysed about one-fifth as fast as acetanilide or one-tenth as fast as *p*-acetamidobenzoic acid at the same concentration. Its apparent K_m was found to be 1·6 mM and it reacted maximally at a rate of 3·7 μmoles/mg. of protein/hr. (cf. Tables 4 and 6). Introduction of a nitro group into the *para* position of the benzamido moiety was without effect on the relative rate; the same substituent in the *meta* position decreased the rate slightly, but in the *ortho* position it reduced the rate by a factor of 50.

Miscellaneous substrates. Other than derivatives of aniline very few compounds were tested as substrates. 2-Acetamidonaphthalene was deacylated about four times as rapidly as acetanilide; the 1-isomer was apparently not attacked. A relatively slow deacylation of 4-acetamidoantipyrin (about 30 % of the rate of acetanilide) was also demonstrated.

Table 5. *Influence of ring substitution on the rate of deacetylation of acetanilide*

Measurements of relative rate were made as outlined in Table 3. The rate of deacetylation of acetanilide is given a value of 100.

Substituent	Relative rate		
	ortho	*meta*	*para*
OH	0·3	38	115
NO_2	25	62	170
$O{\cdot}CH_3$	1·6	—	220
CO_2H	0·2	35	220
CH_3	<0·1	80	330
Cl	15	160	380

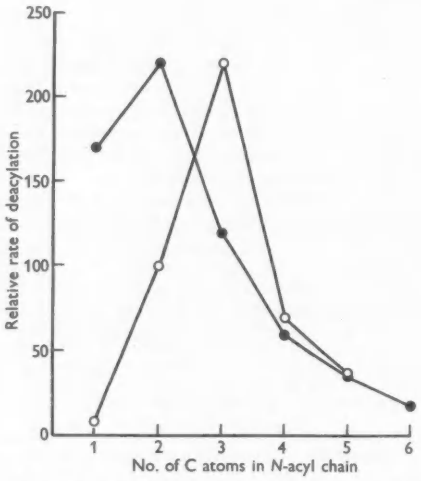

Fig. 6. Effect of *N*-acyl chain length on relative rate of hydrolysis. In the series $R{\cdot}C_6H_4{\cdot}NH{\cdot}CO{\cdot}[CH_2]_n{\cdot}H$ 1 μmole of substrate was incubated with suitably diluted chick-kidney suspension in a total volume of 1 ml. of 0·1 M-phosphate buffer, pH 7·3, for 60 min. at 38°. The substituents R were H (○) and *p*-CO_2H (●).

Table 6. *Effect of* N*-acyl chain length on* K_m *values of derivatives of* p*-aminobenzoic acid and* p*-aminophenylacetic acid*

Values were determined as described in Table 4, in which are given values for substrates with shorter chains. Substrates had the structure $R{\cdot}C_6H_4{\cdot}NH{\cdot}CO{\cdot}[CH_2]_n{\cdot}H$.

R	*n*	K_m	Max. rate
CO_2H	2	1·7	6·4
CO_2H	3	1·3	2·0
CO_2H	4	0·47	1·0
CO_2H	5	0·54	1·5
$CH_2{\cdot}CO_2H$	2	0·70	30
$CH_2{\cdot}CO_2H$	3	0·76	6·8
$CH_2{\cdot}CO_2H$	4	0·15	2·2

Experiments with rat-liver enzyme

Some of the properties of kynurenine formamidase have been described by Mehler & Knox (1950) and by Jakoby (1954). So as to provide some further points of comparison with the chick-kidney enzyme a few more experiments were done. *o*-Formamidobenzoic acid (formylanthranilic acid) was used as a reference substrate.

Inhibitors. Partially purified enzyme was not inhibited by *p*-chloromercuribenzoate at 0·5 mM, the highest concentration tested. At 2 mM-$HgCl_2$, zinc acetate and $CuCl_2$ caused inhibitions of 47, 45 and 9% respectively. It may be recalled (Table 2) that at 2 μM these compounds caused a greater inhibition of the chick-kidney enzyme.

Influence of ring substitution and N*-acyl group.* Formanilide was deacylated at a relatively slow rate; the relative effectiveness of various *ortho* substituents in increasing rate was confirmed and the *o*-chloro derivative was included. The decreasing order of effectiveness was found to be CO_2H, NO_2, Cl, H, CH_3, *o*-methylation resulting in a decrease in rate as compared with formanilide.

It was also confirmed that *para*-substituted compounds were deacylated at a very slow rate; *p*-acetamidobenzoic acid, for example, was hydrolysed at less than 0·02% of the rate of formylanthranilic acid.

As reported by Mehler & Knox (1950), *o*-formamido compounds were hydrolysed considerably faster than the *o*-acetamido derivatives. In the present investigation it was established that the rate increased again with propionylanthranilic acid (Table 7) but thereafter declined with increasing chain length. Chloroacetylation produced a substrate which was deacylated almost as fast as the relative rate reported for formylkynurenine. *o*-Benzamidobenzoic acid was hydrolysed at a very slow, but still measurable, rate.

Table 7. *Effect of different* N*-acyl groups on the hydrolysis of anthranilic acid derivatives by rat-liver enzyme*

Relative rates of reaction were measured as described in Table 3, except that a suitably diluted rat-liver preparation was used.

N-Acyl group	Relative rate
Formyl	100
Acetyl	9
Chloroacetyl	375
Propionyl	27
Butyryl	6
Valeryl	3·5

DISCUSSION

Although kynurenine formamidase and the chick-kidney enzyme both catalyse removal of *N*-acyl groups from substituted acylanilides, there are clear differences between them. The main points of contrast are summarized in Table 8.

From the way in which they were separated it seems reasonable to conclude that most of the particles present in the suspensions used were mitochondria (Hogeboom, Schneider & Palade, 1948; Potter, Recknagel & Hurlbert, 1951; Schneider, 1959). Similarly, most of the microsomes were removed with the supernatant from the second centrifuging. Failure of a variety of treatments to release activity into the 15 000 ***g*** supernatant indicates that the enzyme is very tightly bound to the mitochondria. On the other hand, activity does not depend upon structural integrity of the mitochondria, for this could be severely damaged without great loss of activity. Deoxycholate and Triton X-100, for instance, caused a release of 60–70% of mitochondrial protein yet left nearly all of the original activity in a form still sedimentable at 15 000 ***g***.

A high sensitivity to inhibition by *p*-chloromercuribenzoate, an even higher sensitivity to heavy-metal inhibition, and the partial reversal of the former by glutathione and cysteine all point towards the presence of thiol groups in the region of the enzyme's active site.

Because these experiments were done with relatively crude enzyme preparations (mitochondrial suspensions) it would be unwise to assume that a single enzyme is responsible for the hydrolysis of all the substrates that reacted; on the other hand the simplest hypothesis is to attribute observed activity to but a single enzyme. In this connexion it might be pointed out that the hydrolyses by mitochondrial-bound enzyme of two substrates so different as *p*-acetamidoanisole and *p*-benzamidobenzoic acid have the same pH–activity

Table 8. *Some points of difference between kynurenine formamidase of rat-liver and the chick-kidney deacylase*

	Kynurenine formamidase	Chick-kidney deacylase
Subcellular localization	Cell sap	Mitochondria
Sensitivity to inhibition by metals	Low	High
Optimum *N*-acyl group	Formyl	Acetyl or propionyl
Optimum ring substitution	*ortho*	*para*
Physiological substrate	Formylkynurenine	Unknown

relationships and the same sensitivities to inhibition by *p*-chloromercuribenzoate. Moreover, from the extremely low rate at which *o*-formamidobenzoic acid was hydrolysed, it can be concluded that the deacylation of *ortho*-substituted compounds was not due to the presence of small amounts of kynurenine formamidase. But not until activity has been released into solution and further fractionated can the question of the number of enzymes involved be satisfactorily examined.

For the same reason caution must be used in the interpretation of the kinetic data since diffusion to an active site, rather than formation of a Michaelis–Menten complex, could be a rate-limiting factor. In argument against this might be quoted the high apparent K_m value of formanilide (7·4 mM) as compared with the greater apparent affinities of larger molecules such as *p*-hexanamidobenzoic acid (K_m 0·54 mM) and *p*-benzamidobenzoic acid (K_m 1·3 mM), although this would ignore the possibility of a more rapid diffusion by more highly lipophilic compounds. Whatever the true situation might be, it seems most unlikely that rate-limitation imposed by diffusion can explain the more obvious differences encountered.

More rapid hydrolysis of chloroacetamido than of acetamido derivatives, seen with both chick-kidney and rat-liver enzymes, may be a fairly general phenomenon. It is well known with hydrolysis of *N*-acyl amino acids (Birnbaum, Levintow, Kingsley & Greenstein, 1952) and was noted by Gomori (1954) in hydrolysis of *N*-acyl naphthylamines by human tissues; it is consistent with the readier catalysis of hydrolysis by H^+ and OH^- ions. Yet formamido compounds, even more labile to acid and base hydrolysis, are hydrolysed by chick kidney at maximal rates no greater than those of the acetamido homologues.

Slow rates of hydrolysis of substrates with *ortho* substituents can probably not, at least in the two cases where apparent K_m was measured, be entirely explained by low affinities for the enzyme. Steric hindrance to hydrolysis offered by the *ortho* substituent is another important factor to be taken into account, and may explain the resistance of 1-acetamidonaphthalene to hydrolysis as compared with the 2-isomer. The effect of *ortho* substitution upon activity is in marked contrast with hydrolysis catalysed by kynurenine formamidase, which for optimum conditions demands an *ortho* substituent; it implies a fundamental difference not only between structural arrangements within the active centres but also between the mechanisms of catalysis of the two enzymes. By using a pair of substrates such as *o*-formamidobenzoic acid and *p*-acetamidobenzoic acid the amount of either or both enzymes within a particular tissue can be measured.

A physiological function, if any, for the chick-kidney enzyme is still to be found. A preliminary survey shows it to be absent from the kidney and liver of all mammalian species examined. It is present in varying amounts in the kidney of a variety of birds, particularly in the less-specialized orders, but in some species of the more recent natural orders it has almost disappeared. It has also been found in a high proportion of the reptile species so far examined. Possibly it is a vestigial enzyme surviving from a time when it played a part, no longer important, in the organism's economy. Although it is undesirable to speculate too freely, particularly as a natural substrate may be awaiting discovery, the enzyme may have been acquired at a stage in chordate evolution when some arylamine other than formylkynurenine was of biochemical importance but was in danger of being rendered metabolically inaccessible by increasing powers of acetylation. It is interesting that arylamines can act as acceptors in the liver acetylating system, that activity is higher in pigeon than in mammalian liver and that acceptors of this type include *p*-aminobenzoic acid (Lipmann, 1945; Tabor, Mehler & Stadtman, 1953). No chordate is known which can synthesize its own folic acid from *p*-aminobenzoic acid, but if chordates existed which could perform this synthesis, and if they possessed also a strong acetylating system which accidentally includes arylamines, then acquisition of a mechanism deacetylating the essential metabolite would have been an advantage. In this hypothetical situation loss of the ability to synthesize folic acid would make the deacetylating enzyme redundant and species which lost the enzyme through genetic mutation would be at no selective disadvantage.

SUMMARY

1. Homogenates of chick kidney rapidly deacylate a variety of acylanilides. Activity is associated with the mitochondria, from which it cannot be released by treatment with several surface-active agents or by repeated freezing and thawing. Some properties of the enzyme have been investigated in mitochondrial suspensions.

2. Deacylation is most rapid in phosphate buffer at pH 7·3 and at 44°. Heat of activation has been calculated as 15 300 cal.

3. Heavy metals inhibit the enzyme strongly. At concentrations as low as 0·2 μM, Hg^{2+} ion inhibits by about 20%. Inhibition by *p*-chloromercuribenzoate (50% at 50 μM) is partly reversed by reduced glutathione and cysteine.

4. Substitution in the *para* position of acetanilide generally results in an increase in the relative rate of deacetylation and a decrease in apparent K_m. *Ortho* substitution has a marked effect in the

opposite direction; the decrease in rate (which may be 1000-fold) at moderate substrate concentration can be accounted for only partly by decreased affinity of substrate for enzyme.

5. The effect of increasing the *N*-acyl chain length from formyl to hexanoyl has been tested. Hydrolysis is fastest at acetyl or propionyl, depending upon the nature of the *para* substituent. Of all the substrates tested *p*-acetamidophenylacetic acid has the lowest apparent K_m (78 μM). Chloroacetylated compounds are hydrolysed most rapidly; *p*-chloroacetamidoanisole, for instance, is deacylated at a maximal rate corresponding to 6·1 m-moles/g. of fresh tissue/hr. Some *N*-benzoyl derivatives of *p*-aminobenzoic acid are slowly hydrolysed.

6. On the basis particularly of distribution, subcellular localization, sensitivity to metal inhibition and the effect on rate of ring substitution and *N*-acyl chain length the enzyme is seen to have properties very different from those of kynurenine formamidase.

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REFERENCES

Birnbaum, S. M., Levintow, L., Kingsley, R. B. & Greenstein, J. P. (1952). *J. biol. Chem.* **194**, 455.
Bratton, A. C. & Marshall, E. K. (1939). *J. biol. Chem.* **128**, 537.
Dalgliesh, C. E. (1952). *J. chem. Soc.* p. 141.
Gomori, G. (1954). *Proc. Soc. exp. Biol., N.Y.*, **85**, 570.
Hogeboom, G. H., Schneider, W. C. & Palade, G. E. (1948). *J. biol. Chem.* **172**, 619.
Jakoby, W. B. (1954). *J. biol. Chem.* **207**, 657.
Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
Lipmann, F. (1945). *J. biol. Chem.* **160**, 173.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, F. J. (1951). *J. biol. Chem.* **193**, 265.
Mehler, A. H. & Knox, W. E. (1950). *J. biol. Chem.* **187**, 431.
Potter, V. R. & Elvehjem, C. A. (1936). *J. biol. Chem.* **114**, 495.
Potter, V. R., Recknagel, R. O. & Hurlbert, R. B. (1951). *Fed. Proc.* **10**, 646.
Schneider, W. C. (1959). *Advanc. Enzymol.* **21**, 1.
Shaffer, J. M. & Bieter, R. N. (1950). *J. Pharmacol.* **100**, 192.
Tabor, H., Mehler, A. H. & Stadtman, E. R. (1953). *J. biol. Chem.* **204**, 127.
Venkataraman, A., Venkataraman, P. R. & Lewis, H. B. (1948). *J. biol. Chem.* **173**, 641.

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Purification of a Glycoprotein from Bovine-Submaxillary Glands

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Investigations of the composition of purified mucins from salivary glands have been made by Tanabe (1939), Blix, Svennerholm & Werner (1952) and Blix, Lindberg, Odin & Werner (1956). Degradative procedures were employed in the preparations, since the purpose was to study the composition of the carbohydrate or of the sialic acid. A purified material was prepared by Heimer & Meyer (1956) from mucin clots obtained from extracts of bovine-submaxillary glands. A 6M-urea solution adjusted to pH 9·0 was used for the extraction of acetone-extracted material.

In earlier work from this Laboratory (Nisizawa & Pigman, 1959), mucin clots from bovine-submaxillary glands were freeze-dried as sodium salts. The materials retained the viscosity of the extracts and were soluble in water. The procedure was a modification of the method of Hammarsten (1888). These clots, composed of an anionic glycoprotein combined with a protein cation, precipitated between pH 3·5 and 4·0.

The present work deals with the purification and further investigation of the physical characteristics and chemical composition of the material mainly responsible for the viscous nature of the mucin clot. Two preparations obtained at intermediate stages of purification have also been analysed.

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EXPERIMENTAL

Source material. In the previous work (Nisizawa & Pigman, 1959) the sodium salt of the bovine-submaxillary mucin clot was obtained as follows. Bovine-submaxillary

glands which had been kept frozen were extracted with the dilute phosphate buffer (pH 7·0). The extract was adjusted to pH 3·5 with dilute hydrochloric acid and the resulting mucin clot was dissolved in water by the addition of a small amount of sodium hydroxide. The solution was freeze-dried after extensive dialysis against water. It contained 12·8% of sialic acid (as *N*:*O*-diacetylneuraminic acid), 8·1% of galactosamine and 3·4% of glucosamine (as *N*-acetylhexosamines) and nearly 74% of protein. All work was done at 2–4°. This freeze-dried mucin was the starting material for the present study.

Chemical analyses. For qualitative determination of the sugars, paper chromatography was used after hydrolysis by 1 N-hydrochloric acid or -sulphuric acid in a boiling-water bath for 5 hr. When hydrochloric acid was used the hydrolysates were filtered and dried in a vacuum desiccator over sodium hydroxide and calcium chloride. Barium carbonate was used to neutralize sulphuric acid. The system butanol–pyridine–water (5:3:2) (Yosizawa, 1953) was used as developer, and aniline hydrogen phthalate was used as the colouring reagent (Partridge, 1949). The paper was Whatman no. 1.

Nitrogen was determined by the micro-Kjeldahl method (Clark, 1943). Sialic acid was measured by the modified direct Ehrlich method (Pigman, Hawkins, Blair & Holley, 1958) with *N*-acetylneuraminic acid used as the standard. Hexosamine was analysed by the Elson–Morgan method modified by Boas (1953). For the measurement of glucosamine and galactosamine separately, the method of Gardell (1953) was utilized. Protein was determined by both the biuret reagent (Rosenthal & Cundiff, 1956) and the phenol reagent (Lowry, Rosebrough, Farr & Randall, 1951), crystalline bovine-serum albumin being used as the standard. Fucose (6-deoxyhexose) was measured by the cysteine-sulphuric acid method of Dische (1955). Hexose was determined as galactose by the anthrone method (Hanson, Schwartz & Barker, 1960) after correction for fucose. Uronic acid was measured as glucuronic acid by the Dische carbazole method (Dische, 1955).

Physical measurements. Viscosities were determined in Cannon–Manning Ostwald-type viscometers of about 0·2 ml. capacity at 30°. The flow time for water in the three viscometers was no. 100, 90 sec.; no. 200, 9·9 sec.; no. 300, 3·8 sec. Electrophoretic analyses were conducted at 1° in a Perkin–Elmer model 38 Tiselius-type apparatus with 6 ml. cell. The ascending boundaries were utilized for the calculations of mobility. Ultracentrifugal analyses were made at room temperature by use of the Spinco model E analytical ultracentrifuge at 259 700 *g* with a 12 mm. cell.

pH. All pH values were measured with a Beckman model G pH meter and glass electrode.

Purification with ethanol. To 100 ml. of aqueous 2% solution of mucin clot, 11 ml. of 50% calcium chloride solution was added at 2–4°; absolute ethanol was then added slowly with mechanical stirring in an ice–water bath. The precipitate produced at 30% (v/v) ethanol concentration was removed by centrifuging at 26 360 *g* for 15 min. and discarded. Additional ethanol was added to the supernatant to give a final concentration of 60%. After similar centrifuging, this precipitate was suspended in 30 ml. of water. Insoluble portions were nearly completely dissolved by the addition of a 4% solution of sodium ethylenediaminetetra-acetate, pH 9·5. The solution was then dialysed against two changes of 2 l. of water for 3 days with stirring. A small precipitate produced during dialysis was centrifuged off. The mucin solution contained 247 mg. of protein/100 ml. (biuret method) and 110 mg. of sialic acid/100 ml. The yields were 19 and 50% respectively. As the solution was diluted during dialysis it was concentrated to half its volume by dialysis against a polyvinylpyrrolidone solution. This product was called F1.

Purification by Sevag (1934) *procedure and with ethanol.* The mucin clot (0·4 g.) was dissolved in 30 ml. of water with 3·16 g. of calcium acetate at 2–4°, and 20 ml. of 0·5 M-acetic acid was added. The resulting slightly turbid solution was shaken mechanically with 100 ml. of amyl alcohol–chloroform (1:3, v/v) for several minutes and then was separated from the solvent by centrifuging. The procedure was repeated many times until no interfacial precipitate formed. Ethanol was added to the deproteinized solution in an ice–salt bath with stirring to give a final concentration of 40% (v/v) of ethanol. The resulting precipitate was removed by centrifuging at 26 360 *g* for 15 min. and was suspended in 3–4 ml. of water and centrifuged.

A number of solutions obtained in this way from a total of 3·5 g. of mucin clot were combined to give 50 ml. To the combined solution was added a little 5% sodium ethylenediaminetetra-acetate, pH 6·7, and the solution was dialysed for 3 days against water. The solution was adjusted to pH 8·5 by adding a little 2% sodium carbonate solution and the solutions were centrifuged at 26 360 *g* for 20 min. The resulting solution was product F2. The yield was 3%, based on the original dry weight of mucin clot.

The supernatant of a 40% ethanol solution from the preparation of product F2 was diluted with ethanol to 60% ethanol (v/v). The precipitate was separated and redissolved in water, and the solution was treated with sodium ethylenediaminetetra-acetate and dialysed as for product F2. The resulting 50 ml. from 3·5 g. of mucin clot contained product F3. Its yield was 10% of the original dry mucin clot.

RESULTS

Mucin clot of the previous work from the neutral cold extracts of bovine-submaxillary glands was purified by precipitation with ethanol in the presence of calcium chloride or acetate and by the Sevag procedure. The physical properties and chemical analyses of these products are given below. Three products were isolated at different stages of the purification. These are designated as F1, F2 and F3, in increasing order of purification.

Mucin-clot formation. Aqueous solutions of F1 and F2 became turbid when adjusted to pH 3·5 with dilute hydrochloric acid, and formed a typical clot only if serum albumin had been added previously. When albumin was added to a very dilute solution of F3 before acidification, only a turbidity appeared at pH 3·5. However, the turbid solution deposited a viscous mass at the bottom of the test tube after 18 hr. at room temperature.

Viscometry. The viscosity of product F1 was measured in a phosphate buffer, pH 7·4, I 0·1, at 30°. The viscosity was 4·64 centipoises for a 1·2% solution (dry-weight basis). This corresponded to

53% of the viscosity of the original mucin clot when the products were compared on the basis of an equal amount of sialic acid.

The viscosity of an aqueous 0·72% solution of mucin F3 at 30° was measured in three Cannon–Manning capillary viscometers of quite different rates of flow (nos. 100, 200 and 300). The viscosity was 18·8 centipoises (viscosity, relative to water, 22·1), and no effect of flow rate was noticeable. This product (F3) was degraded by drying at 100° for several hours, and the viscosity dropped from 18·8 to 12·1 centipoises.

The viscosity of F3 was measured at different concentrations in water and in phosphate buffers of pH 7·4 (I 0·1 and 0·2). The viscosity of the original mucin clot was also measured under the same conditions. These results are shown in Figs. 1 and 2. The intrinsic viscosity of mucin clot was 26·3 in water and 2·0 and 1·7 at I 0·1 and 0·2 respectively. It was 64·6, 3·9 and 3·6 respectively for product F3.

Electrophoretic behaviour. In the work by Nisizawa & Pigman (1959) the mucin clot from bovine-submaxillary glands separated into several components during electrophoresis at pH 10·0, whereas the resolution was poorer at pH 7·0–8·0. Therefore the electrophoretic patterns were studied at pH 10·0–11·0 in the present work. Fig. 3 shows the electrophoretic pattern of F1 at pH 10·0. The fast-moving component with a mobility about -16×10^{-5} cm.^{2} v^{-1} sec.$^{-1}$, present in the original mucin clot (Nisizawa & Pigman, 1959), was not detectable, but the material still showed several components. In the electrophoretic pattern of product F2, two components were still evident. Their mobilities were $-6{\cdot}7$ and $-16{\cdot}5 \times 10^{-5}$ cm.^{2} v^{-1} sec.$^{-1}$. These mobilities corresponded to those of the most slowly moving and the most rapidly moving components in the original mucin clots.

In Fig. 3, only a single peak can be seen for product F3, and its mobility was $-6{\cdot}9 \times 10^{-5}$ cm.^{2} v^{-1}

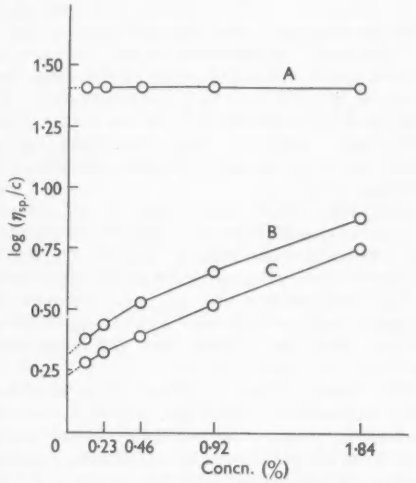

Fig. 1. Intrinsic viscosity of mucin A in different solvents at 30°: A, water; B, phosphate–NaCl buffer, pH 7·4, I 0·1; C, phosphate–NaCl buffer, pH 7·4, I 0·2.

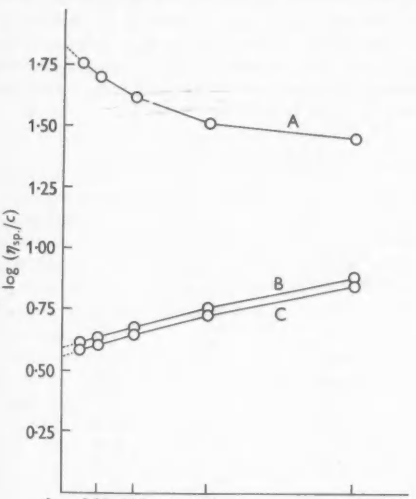

Fig. 2. Intrinsic viscosity of product F3 in different solvents at 30°: A, water; B, phosphate–NaCl buffer, pH 7·4, I 0·1; C, phosphate–NaCl buffer, I 0·2.

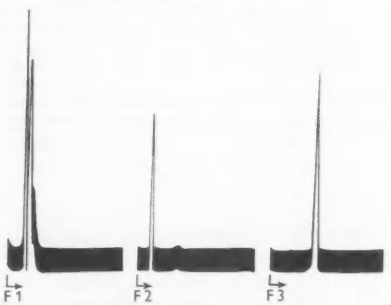

Fig. 3. Electrophoretic patterns: F1 in phosphate–NaCl buffer, pH 10·0, I 0·1, at 70 min. Concentration: 1·23%. Mobilities of components: $-6{\cdot}6$, $-7{\cdot}6$, $-8{\cdot}7$ ($\times 10^{-5}$ cm.^{2} v^{-1} sec.$^{-1}$). F2 in phosphate–NaCl buffer, pH 10·0, I 0·1, at 40 min. Concentration: 0·2%. Mobilities of components: $-6{\cdot}7$, $-16{\cdot}5$ ($\times 10^{-5}$ cm.^{2} v^{-1} sec.$^{-1}$). F3 in phosphate–NaCl buffer, pH 11·0, I 0·1, at 120 min. Concentration: 0·5%. Mobility $-6{\cdot}9$ ($\times 10^{-5}$ cm.^{2} v^{-1} sec.$^{-1}$).

sec.$^{-1}$ under the same conditions. This component corresponds to one of the principal ones observed in the patterns of the original clot (−7·4) and seems to be mainly responsible for the viscosity of the extracts of the gland. The single fractionation with ethanol mainly removed the fastest-moving component, and the Sevag procedure seemed to remove the protein components with intermediate mobilities.

Optical rotation. The specific optical rotation of F3 was measured in a 2 dm. tube as $[\alpha]_D^{24}$ −3·5 (1·44 % in water).

Ultracentrifugal characteristics. The ultracentrifugal patterns of products F1 and F3 are shown in Fig. 4. In the pattern of F1, two major peaks and one minor peak were still present, as in that of the original mucin clots. The pattern of F3 also showed two peaks, whereas its electrophoretic pattern showed a single peak. However, the slower peak in the pattern of F3 represented less than 10 % of the area. The $S_{20,w}$ for the three peaks in F1 are 2·15, 2·41 and 3·07 respectively and 1·88 and 2·28 for the two components in F3. The $S_{20,w}$ of the corresponding peaks are sufficiently close to assume that they represent the same components in both preparations. However, four components were found in the electrophoretic pattern of F1, whereas F3 showed a single peak. From the area of the peaks, however, the faster (2·3 s) component in Fig. 4 seems to represent the major substance in product F3.

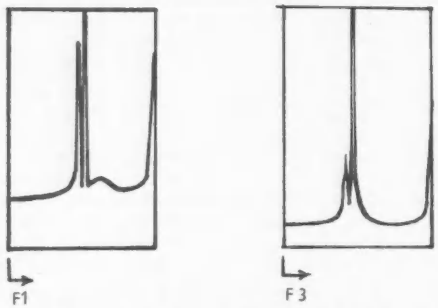

Fig. 4. Ultracentrifugal patterns of products F1 and F3 in phosphate buffer, pH 10·0, I 0·1. F1: concentration, 1·2 %; time, 120 min. F3: concentration, 0·5 %; time, 160 min.

Chemical analyses. The results of the chemical analysis of the three products are given in Table 1. The ascending order of purity was F1, F2, F3.

The purified preparation F3 contained about 38 % of protein and 62 % of carbohydrate (comprising 32·3 % of sialic acid and 27·3 % of hexosamine). Minor components were galactose, fucose and hexuronic acid.

The less-pure product F2 consisted of almost equal amounts of protein and carbohydrates. The amount of hexosamine was larger than that of sialic acid, and galactosamine and glucosamine were found in almost equal amount.

The molar ratio of galactosamine to sialic acid was almost exactly unity for both F2 and F3. Some of the glucosamine in F2 seemed therefore to originate from a component other than that which was composed of an equal amount of galactosamine and sialic acid. F2 contained 6 % of hexuronic acid and about 10 % of glucosamine. Since sulphate was absent, this composition suggested the presence of some hyaluronic acid.

Table 1. *Chemical analyses of submaxillary mucin at different stages of purification*

Compositions are given on a dry-weight basis without correction for ash. The ester sulphate test on F2 was negative for a sample of 17 mg. dry wt. No ash was detected when a sample of F3 was ignited in a platinum crucible. On paper chromatograms of hydrolysates of mucin A and F3, definite spots for galactose, galactosamine and glucosamine were found. Spots corresponding to glucose and mannose were not detected, but those of fucose were found in traces when sulphuric acid was used for hydrolysis.

Constituent	Composition (%) F1	F2	F3
Total nitrogen	11·9	9·6	9·4
Total N minus non-protein N (corr. total N)	10·1	8·7	6·0
Corr. total N × 6·25*	62·5	47·7	37·5
Biuret protein (as serum albumin)	43·1	20·9	34·1
Phenol-reagent protein (as serum albumin)	—	38·5	32·5
Sialic acid [as *O*:*N*-diacetyl, 'direct' Ehrlich reagent method (see Whelan, 1957)]*	17·3	15·4	32·3
Hexose (as galactose, corr. for fucose)*	3·2	2·5	1·7
6-Deoxyhexose (cysteine–H_2SO_4 method, as fucose)*	—	1·2	0·7
Hexuronic acid (as glucurone)*	0·1	6·0	0·7
Hexosamine (as *N*-acetylglucosamine)*	16·2	21·0	27·3
N-Acetylgalactosamine	—	10·7	21·6
N-Acetylglucosamine	—	10·3	5·7
Total (of items marked *)	99·3	93·8	100·2

DISCUSSION

The mucin clot obtained from the extracts of bovine-submaxillary glands and which contained the viscous material of the extracts was further fractionated. By removing more than half of the protein from the clot by the Sevag procedure and a subsequent ethanol fractionation, a material (F 3) principally responsible for the viscous nature of the clot has been prepared in a purified state.

This material (F 3) had an intrinsic viscosity of 3·6 in a phosphate buffer of pH 7·4, I 0·2 (Fig. 2). The intrinsic viscosity of the original mucin clot was 1·70 under the same conditions (Fig. 1). These intrinsic viscosities were much lower than those for the aqueous solutions and apparently are an example of the well-known electroviscous effect (Overbeek & Bungenberg de Jong, 1949). Although the intrinsic viscosity was doubled as a result of purification, the purity of F 3 was approximately three times that of the starting mucin on the basis of the sialic acid or the galactosamine content.

The intrinsic viscosity of solutions of F 3 in pure water was nearly three times that of a similar solution of the mucin clot, and the plots of log $(\eta_{sp.}/c)$/concentration were quite different (Figs. 1 and 2). The line for the clot was straight and seemed virtually independent of concentration, but that for the purified material was a more normal curve rising with a decrease in concentration (Overbeek & Bungenberg de Jong, 1949). These effects may have arisen in part from a change in the pH with concentration, since an aqueous 0·72 % solution was pH 4·5 and a 0·1 % solution was pH 6·0. In contrast, the solution of mucin clot in water remained in the range pH 6·0–6·8 upon dilution. However, the additional protein of the mucin clot may be a factor in the unusual constant viscosity of mucin clot.

The purified material (F 3) showed one main peak in the ultracentrifugal pattern (Fig. 4), and a single peak in the electrophoretic pattern (Fig. 3). The small peak in the ultracentrifugal pattern and the small amounts of glucosamine, hexose and fucose suggest that the material still is not completely homogeneous.

A principal component of the submaxillary mucin is thus a viscous substance apparently composed of about one-third of peptide units and equimolar proportions of galactosamine and sialic acid. Since the material showed a single peak in the electrophoretic pattern, even at pH 11·0, the linkage between carbohydrate and protein moieties seems to be firm, probably covalent. The product appears to belong to the glycoprotein group (Pigman, 1957).

This material is similar to that obtained by Heimer & Meyer (1956) and has a similar optical rotation. However, the earlier product contained slightly less sialic acid and galactosamine although described as electrophoretically and ultracentrifugally homogeneous. The molar ratio of galactosamine to sialic acid was close to unity in their product also.

A less-purified material (F 2) was richer in glucosamine, galactose and fucose. The molar ratio of glucosamine to the galactosamine was as great as 0·4, even after the glucosamine content was corrected for the contribution by a presumed hyaluronic acid (based on the hexuronic acid content). The same ratio obtained for the more purified product (F 3) was nearly 0·25. Nevertheless, the molar ratios of galactosamine to sialic acid were exactly unity in both F 2 and F 3. These facts suggest the presence of additional glycoproteins in addition to hyaluronic acid and the principal one comprised of equimolar amounts of galactosamine and sialic acid.

SUMMARY

1. The mucin clots obtained from the extracts of bovine-submaxillary glands have been fractionated and purified. The material mainly responsible for the viscous nature of the extract has been obtained purified by the Sevag procedure and by fractionation with ethanol. It showed a single component in the electrophoretic pattern, but a small component separated from the major one in the ultracentrifugal run. The electrophoretic mobility of the product was $-6{\cdot}9 \times 10^{-5}$ cm.^{2}v^{-1} sec.$^{-1}$ in phosphate buffer, pH 11·0, I 0·1. The sedimentation constant of the major component was 2·28 in phosphate buffer, pH 10·0, I 0·1.

2. This material appeared to be a glycoprotein composed of about 38 % of protein and 62 % of carbohydrate. Of the latter, sialic acid was 32·3 % (as the diacetyl compound) and the hexosamines were 27·3 % (as the acetyl compound). The hexosamines contained 21·6 % of galactosamine and 5·7 % of glucosamine. The molar ratio of galactosamine to sialic acid was unity, as in the original mucin clots.

3. A less-purified product obtained from the mucin clots after the treatment with the Sevag procedure appeared to contain some hyaluronic acid. Although the glucosamine content was nearly equal to the galactosamine, the molar ratio of galactosamine to sialic acid still was unity.

4. In view of the electrophoretic and chemical analyses, it was established that the most slowly moving component in the electrophoretic patterns at pH 10·0 represented the principal component responsible for the viscous nature of the mucin and that the product is probably a glycoprotein.

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REFERENCES

Blix, G., Lindberg, E., Odin, L. & Werner, I. (1956). *Acta Soc. Med. Upsalien.* **61**, 1.
Blix, G., Svennerholm, L. & Werner, I. (1952). *Acta chem. scand.* **6**, 358.
Boas, N. F. (1953). *J. biol. Chem.* **204**, 553.
Clark, E. P. (1943). *Semimicro Quantitative Analysis*, 1st ed., p. 37. New York: Academic Press Inc.
Dische, Z. (1955). *Method of Biochemical Analysis*, 1st ed., vol. 2, p. 343. New York: Interscience Publishers Inc.
Gardell, S. (1953). *Acta chem. scand.* **7**, 207.
Hammarsten, O. (1888). *Hoppe-Seyl. Z.* **12**, 163.
Hanson, R. W., Schwartz, H. & Barker, S. B. (1960). *Amer. J. Physiol.* (in the Press).
Heimer, R. & Meyer, K. (1956). *Proc. nat. Acad. Sci., Wash.*, **42**, 728.
Lowry, O. M., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
Nisizawa, K. & Pigman, W. (1959). *Arch. oral. Biol.* **1**, 161.
Overbeek, J. Th. G. & Bungenberg de Jong, H. G. (1949). *Colloid Science*, vol. 2, p. 184. Ed. by Kruyt, H. R. New York: Elsevier Publishing Co.
Partridge, S. M. (1949). *Nature, Lond.*, **164**, 443.
Pigman, W. (1957). *The Carbohydrates*. New York: Academic Press Inc.
Pigman, W., Hawkins, W. L., Blair, M. G. & Holley, H. L. (1958). *Arthritis and Rheumatism*, **1**, 151.
Rosenthal, H. L. & Cundiff, H. I. (1956). *Clin. Chem.* **2**, 394.
Sevag, M. G. (1934). *Biochem. Z.* **273**, 419.
Tanabe, Y. (1939). *J. Biochem., Tokyo*, **30**, 181.
Whelan, W. J. (1957). *Rep. Progr. Chem.* **54**, 321.
Yosizawa, Z. (1953). *Tohoku J. expt. Med.* **59**, 1.

Biochem. J. (1960) **75**, 298

The Glycogenolytic Activity of some Substituted Salicylic Acids

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Sodium salicylate in moderate or large dosage has been shown to decrease glycogen concentration in the liver of fed and starved mice 2 hr. after injection (Sproull, 1954) and in rats 4–7 hr. after administration (Lutwak-Mann, 1942; Smith, Meade & Bornstein, 1952; Feeney, Carló & Smith, 1955). Lutwak-Mann (1942) studied the effect of large doses of certain substituted benzoic acids on rat-liver glycogen, and found that although acetylsalicylate, benzoate, anthranilate, *o*-cresotate (2-hydroxy-3-methylbenzoate) and *m*-hydroxybenzoate all caused some decrease in liver-glycogen concentration 7 hr. after injection, only acetylsalicylate and *o*-cresotate showed an effect similar to salicylate; with *o*-cresotate (0·6 mg./g.) there was virtually no glycogen present over the 24 hr. period of the experiment. Feeney *et al.* (1955) reported that salicylate, acetylsalicylate and *m*-hydroxybenzoate all decreased rat-liver-glycogen concentration significantly although the reduction after *m*-hydroxybenzoate was not as pronounced as that obtained with salicylate.

The present investigation was to determine which of ten substituted benzoic acids related to salicylic acid and of therapeutic interest in the treatment of rheumatic fever decreased the liver-glycogen concentration of fed mice 2 hr. after injection.

METHODS

Swiss albino mice were used in all experiments and the drugs were all given by intraperitoneal injection. The mice were killed by cervical dislocation 2 hr. after injection and 300–400 mg. of liver was removed for the estimation of liver glycogen. Glycogen was separated and hydrolysed by the Good, Kramer & Somogyi (1933) modification of Pflüger's method, and finally it was estimated as glucose by ceric sulphate titration (Miller & van Slyke, 1936). The glycogen concentration was expressed as mg./g. wet wt. of liver.

In the first experiments mice (age 3–5 months) were dealt with in batches of nine of the same sex per day, and were allowed food and water freely until the start of the experiment. Three animals from each batch were killed without injection at zero time, another three were injected with 0·2 ml. of 0·9% NaCl soln. and the remaining three received the same volume of the test drug, these two last-named trios being killed 2 hr. after injection. Four such batches, two for each sex, were used for each drug, which was given as a solution of the sodium salt (pH 7·0–9·0).

Dose–response curve for salicylic acid. Mice (age 2–4 months) of weight range 30–50 g. were allowed food and water until the start of the experiment. The mice were treated in batches of seven mice of the same weight and sex; each of six mice received a dose of 2, 4, 6, 8, 10 or 12 mg. of salicylic acid as a solution of the sodium salt (pH 6·0–8·0) and the seventh mouse received 0·05 ml. of 0·9% NaCl soln. The order in which the doses were given was determined from a table of random numbers. Sixteen estimates (eight males, eight females) of ΔG, i.e. the differ-

ence in liver glycogen between the corresponding control and treated mice, were determined for each dose.

Parallel-line assay. The compounds used, each in four doses, were salicylic acid, *o*-cresotic acid, *m*-cresotic acid (2-hydroxy-4-methylbenzoic acid), *p*-cresotic acid (2-hydroxy-5-methylbenzoic acid) and 3-phenylsalicylic acid, made up as solutions of their sodium salts. Mice (age 3–5 months) of weight range 30–50 g. were treated in batches of 12 of the same sex. Two mice were given 0·05 ml. of 0·9 % NaCl soln.; each of the other ten received one of four doses of each of the five drugs. The four doses of the five drugs were chosen so that the doses of each drug were evenly spaced on a logarithmic scale and the range of expected responses to the drugs was approximately the same. The order of observations with respect to drug and dose was randomized. Twelve estimates of ΔG, i.e. the difference in liver-glycogen concentration between the mean of the two controls and the treated mice within the same batch, were made for each dose and drug for both sexes. Sixteen hours before the start of the experiment the drinking water was replaced by 5 % glucose solution; food was allowed until the start of the experiment.

RESULTS

Table 1 shows the effect of ten compounds on the liver-glycogen concentration of normal, fed mice. The compounds which decreased the liver-glycogen concentration were salicylic acid, *o*-, *m*- and *p*-cresotic acid and 3-phenylsalicylic acid. The mean liver-glycogen concentration of 100 control mice killed 2 hr. after injection of 0·9 % NaCl soln. was $25·3 \pm 1·5$ mg./g. (σ 14·7). The liver-glycogen concentrations were consistent with a normal distribution and a quality-control chart of batch means confirmed that the experiment was under statistical control.

Lutwak-Mann (1942) took as her controls the liver-glycogen concentration of those rats killed at zero time. The present experiments show that, with mice, this practice would lead to erroneous conclusions. The mean glycogen concentration of 90 mice killed at zero time was 41·0 mg./g. This mean was significantly higher than the value 2 hr. after injection of 0·9 % NaCl soln. (*t* test, $P < 0·001$). The liver-glycogen concentrations of 90 mice killed at zero time also showed an approximately normal distribution and the quality-control chart for these results was acceptable.

Dose–response curve for salicylic acid. The log dose–response curve for the effect of salicylic acid on mouse-liver-glycogen concentration (Fig. 1) showed an approximately linear relationship between log dose and ΔG. Homogeneity of variance was confirmed by Bartlett's test (1937), $P > 0·50$. Table 2 gives the analysis of variance of ΔG over the dose range used, which shows that there was no significant component of variance due to deviations from the linear regression found when the dose was transformed into a logarithmic scale. The mean liver-glycogen concentration of the 16 control mice was $27·6 \pm 2·8$ mg./g. (σ 11·3).

Parallel-line assay. The form of the dose–response curve for salicylic acid suggested that the potencies of the other glycogenolytic drugs relative to salicylic acid could be determined by a comparative parallel-line assay.

Before proceeding to this experiment, attempts were made to reduce the variance of the glycogen concentration. Sproull (1954) noted that the variance of male mice was greater than that of females. In the present investigation the coefficient of variation for the control males was 64 %; for the females it was 45 %. Replacing the drinking water by 5 % glucose solution 16 hr. before the start of the experiment slightly reduced these coefficients to male 48 %, female 23 %, through increases in the mean liver-glycogen concentrations rather than through a reduction in variance. The mean glycogen concentration of 84 male control mice killed 2 hr. after the injection of 0·9 % NaCl soln. was $24·9 \pm 1·7$ mg./g. (σ 15·9), and for 86 female mice it was $29·9 \pm 1·4$ mg./g. (σ 13·4). The mean for 50 control male mice given 5 % glucose to drink 16 hr. before the start of the experiment was $33·3 \pm 2·6$ (σ 16·0), and for 50 female mice was $47·5 \pm 1·5$ mg./g. (σ 10·8).

Table 1. *Liver glycogen of treated and control mice killed 2 hr. after injection*

Each value is the mean of 12 determinations of liver glycogen. $P > 0·05$ is regarded as not significant.

Compound	Dose (mg.)	Mean concn. of liver glycogen (mg./g.) Control	Treated	*P*
Salicylic acid	10	21·43	7·39	<0·01
o-Cresotic acid	10	26·12	5·40	<0·01
m-Cresotic acid	10	34·01	9·23	<0·001
p-Cresotic acid	10	22·94	9·29	<0·02
3-Phenylsalicylic acid	6	22·24	10·82	<0·01
Gentisic acid	10	29·33	29·17	>0·5
γ-Resorcylic acid	10	35·62	30·68	>0·10
p-Hydroxybenzoic acid	10	29·22	21·56	>0·10
m-Hydroxybenzoic acid	10	19·33	14·40	>0·10
Salicylamide	10	32·48	24·27	>0·10

For the mice given 5% glucose to drink there was a significant difference in variance between the males and females (F ratio 2·19, $P < 0·01$). The mean of the females was also significantly higher than that of the males (t test, $P < 0·001$). For both sexes the glycogen concentrations showed approximately normal distributions (Fig. 2*B*), and quality-control charts of means of three consecutive estimates of liver-glycogen concentration 2 hr. after injection with 0·9% NaCl soln. showed that the experiments were under statistical control.

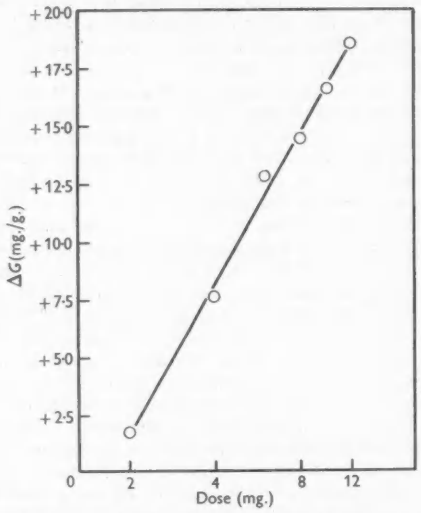

Fig. 1. Relation between log dose of salicylic acid and ΔG (difference in liver-glycogen concentration between corresponding control and treated mice). Points represent the mean of 16 determinations (eight males, eight females) of ΔG and the line is drawn in accordance with the regression equation $Y = 21·76X - 4·98$, where Y is ΔG (mg./g.) and X is log dose (mg.).

Table 2. *Analysis of variance of ΔG and the regression equation of ΔG on log dose for salicylic acid*

Regression equation is $Y = 21·76X - 4·98$, where $Y = \Delta G$ (mg./g.), i.e. difference in liver-glycogen concentration between corresponding control and treated mice, and X is log dose (mg.).

Source of variance	Degrees of freedom	Mean squares
Between doses		
Due to linear regression of ΔG on log dose	1	3136
Deviation from linear regression	4	4
Within doses	90	212

Since there was a significant difference in variance between the sexes, the male and female mice were dealt with separately. For both sexes the high variance of the results masked any relationship between dose and response for *m*- and *p*-cresotic acid. Salicylic acid, *o*-cresotic acid and 3-phenylsalicylic acid all showed an approximately linear relationship between log dose and ΔG over the dose ranges used (Fig. 3). Table 3 gives the doses used and the regression equations for both sexes.

The requirements for a valid comparative parallel-line assay are linearity and parallelism. Homogeniety of variance was confirmed for both sexes by Bartlett's test (females $P > 0·50$, males $P > 0·10$); analyses of variance then showed that there was no significant deviation from log-linear regression, nor was there a significant component of variance due to differences in slope of the regression lines (Table 4).

The molar potency ratios of *o*-cresotic acid and 3-phenylsalicylic acid as glycogenolytic agents relative to salicylic acid were calculated and are shown with their 95% fiducial limits, obtained from Fieller's (1940) theorem, in Table 5. For both sexes, 3-phenylsalicylic acid was significantly more potent than salicylic acid; the potency ratios were 4·79 in the females and 2·68 in the males. Although the potency ratios for *o*-cresotic acid were also greater than 1 in both sexes only the females showed a significant effect. The 95% fiducial limits were wide, owing to the very high variance of

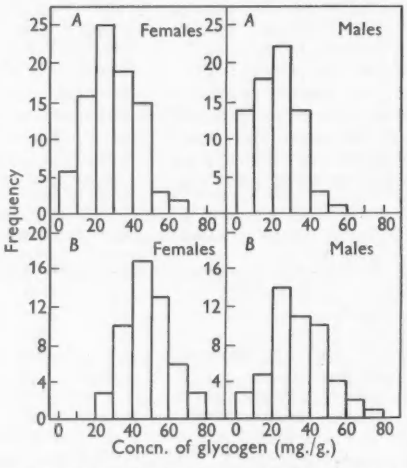

Fig. 2. Frequency distributions of control liver-glycogen concentrations of male and female mice given (*A*) water to drink, and (*B*) 5% glucose 16 hr. before the start of the experiment.

Table 3. *Regression equations, for both sexes, of ΔG upon log dose, and dose range for the three compounds used in the parallel-line assay*

$Y = \Delta G$, i.e. difference in liver-glycogen concentration between the mean of the two controls and the treated mice from the same batch (mg./g.); X = log dose (mg.).

Compound	Dose range (mg.)	Regression equation	
		Female	Male
Salicylic acid	4–12	$Y = 20{\cdot}4X - 7{\cdot}0$	$Y = 20{\cdot}9X - 1{\cdot}3$
o-Cresotic acid	3·04–8	$Y = 28{\cdot}2X - 7{\cdot}5$	$Y = 37{\cdot}5X - 10{\cdot}1$
3-Phenylsalicylic acid	2·04–6	$Y = 36{\cdot}2X - 4{\cdot}1$	$Y = 39{\cdot}6X - 7{\cdot}2$

Table 4. *Parallel-line assay: general analysis of variance*

		Female	Male
Adjustment for mean		22 620·16	34 292·87
Source of variance	Degrees of freedom	Mean squares	Mean squares
Preparations	2	356	51
Regression	1	3 313	4 321
Parallelism	2	94	155
Deviation from log-linear regression	6	65	155
Residual (within doses)	132	137	248

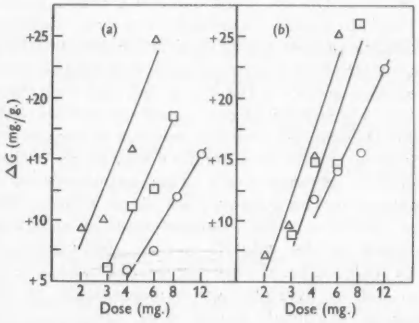

Fig. 3. Relation between dose of the drug and ΔG (difference between the mean of the two control mice and the treated mice from the same batch) for (*a*) females and (*b*) males. The dose is plotted on a logarithmic scale and points represent the mean of 12 determinations of ΔG. ○, Salicylic acid; □, *o*-cresotic acid; △, 3-phenylsalicylic acid.

Table 5. *Molar potency ratios of o-cresotic acid and 3-phenylsalicylic acid with respect to salicylic acid as glycogenolytic agents in male and female mice*

Figures in parentheses represent the 95% fiducial limits.

Compound	Molar potency ratios	
	Females	Males
o-Cresotic acid	1·81 (1·22–2·84)	1·50 (0·89–2·48)
3-Phenylsalicylic acid	4·79 (2·93–8·31)	2·68 (1·50–4·33)

the liver-glycogen concentrations. The merits of any assay can be decided from 'g', the index of precision, defined as $(t^2s^2)/(b^2\Sigma Sxx)$ where s^2 is the variance and Sxx is $S(x-\bar{x})^2$ (Finney, 1952). The high values of 'g' (female 0·16, male 0·22) showed that this was not a satisfactory assay.

DISCUSSION

The wide variation in liver-glycogen concentration of mice seems unavoidable. Venning, Kasmin & Bell (1946) and Nissim (1953), working on the assay of glucocorticoid hormones by the mouse-liver-glycogen method, experienced the same difficulties, and Nissim (1953) stated that the liver-glycogen concentration of mice was sensitive to changes in temperature, feeding, environment and purity of strain. Barnes (1953) reported the same variation in rats and reduced it slightly by submitting the animals to regular feeding regimes. The practice of regular feeding times is impracticable in the mouse, but replacement of the drinking water by 5% glucose solution decreased the coefficient of variation by increasing the mean liver-glycogen concentration. The coefficients of variation in the present assay were, however, even without supplementary glucose, much smaller than those quoted by Nissim (1953), who gave a value of 78% for the coefficient of variation of liver-glycogen concentrations of normal, fed mice. The excessive frequency of very low liver-glycogen concentrations in the males was diminished in the present investigation by the extra glucose in the water (Fig. 2). It was, however, necessary to use large samples, and the values of 'g' were still higher than desirable.

Nissim (1953) reported that liver-glycogen concentrations of control mice did not follow a normal distribution. In the present investigation liver-glycogen concentrations of both sexes were consistent with normal distributions (Fig. 2*A* and *B*), and quality-control charts confirmed that the experiments were under statistical control, whether or not the animals received a supplement of 5% glucose in their drinking water.

The drugs that, in 10 mg. doses, did not alter the concentration of glycogen in mouse liver were *m*- and *p*-hydroxybenzoic acid, gentisic acid, γ-resorcylic acid and salicylamide. Lutwak-Mann (1942), using higher doses in rats (0·8 mg./g.), claimed that over a 24 hr. period *m*-hydroxybenzoate caused a gradual decrease in the liver-glycogen concentration; she did not examine the other compounds.

The only compounds which decreased the liver-glycogen concentration of fed mice were salicylic acid, *o*-, *m*- and *p*-cresotic acid and 3-phenylsalicylic acid. All but the 3-phenylsalicylic acid have been used therapeutically in the treatment of rheumatic fever (May, 1909; Stockman, 1912).

The dose–response curve for salicylic acid was linear when ΔG was plotted against dose transformed into a logarithmic scale. Inspection of the results indicated that on the days when the control values were low the treated mice also tended to give lower results; ΔG was therefore used rather than the actual glycogen concentration. The variance of ΔG within each dose and drug was also slightly lower than the variance of the glycogen concentration, suggesting that use of the difference between the control and treated mice within the same batch reduced some of the variation attributable to environmental conditions.

A standard comparative parallel-line assay was used for the determination of potency. Both 3-phenylsalicylic acid and *o*-cresotic acid were more powerful glycogenolytic agents than salicylic acid when administered in single doses intraperitoneally to mice. 3-Phenylsalicylic acid was apparently the more powerful (Table 5). The fiducial limits showed that the differences were significant at the 95% level for both drugs in the females but only for 3-phenylsalicylic acid in the males. For both drugs this difference in potency between the sexes could not be definitely established from the present assay and is probably largely fortuitous.

One of the many biological properties of salicylic acid is its ability to increase the rate of oxygen consumption in man (Barbour & Devenis, 1919) and animal (Singer, 1901; Reid, 1957). *o*-, *m*- and *p*-Cresotic acid and 3-phenylsalicylic acid have a similar stimulant action in the rat; the relative molar potency ratios of these drugs compared with salicylic acid were: *o*-cresotic acid 2·6, *m*-cresotic acid 1·8, *p*-cresotic acid 1·9 (Andrews, 1958) and 3-phenylsalicylic acid 7·5 (M. M. Andrews, unpublished result). In the present series only those compounds which increased the rate of oxygen consumption of rats possessed glycogenolytic activity. The present results do not exclude a relationship between glycogenolytic and metabolic activity. In mice 3-phenylsalicylic acid shows the greatest glycogenolytic effect, followed by *o*-cresotic acid, *m*- and *p*-cresotic acid and salicylic acid. This ranking also applies to the effects of these compounds as metabolic stimulants in rats (Andrews, 1958). The molar potency ratios of *o*-cresotic acid and 3-phenylsalicylic acid as glycogenolytic agents relative to salicylic acid were of the same order as those for metabolic activity. Other metabolic stimulants have also been found to lower the liver-glycogen concentration. Barnes (1953) found that single doses of dinitro-*o*-cresol, a powerful metabolic stimulant, decreased the liver-glycogen concentration of rats. Thyroid hormones, which also increase metabolic rate, possess glycogenolytic properties in animals (Cramer & Krause, 1913; Houssay, 1946). Cramer & McCall (1917) suggested that the 'increased oxidative processes in the cell' after the administration of thyroid hormones were due to the glycogenolytic action of the hormones, and Sutherland (1951) has supported the suggestion of Cori (1931) that at least part of the increased oxygen consumption caused by adrenaline may be the indirect result of glycogenolysis. This hypothesis seems unlikely in the present case since Meade (1954) and Andrews (1958) demonstrated a significant increase in the rate of oxygen consumption of rats within 1 hr. of injecting salicylic acid, whereas the fall in liver-glycogen concentration in rats became apparent about 4 hr. after injection (Lutwak-Mann, 1942).

SUMMARY

1. The effects of ten substituted salicylic acids on liver-glycogen concentration in the mouse have been studied. Doses (10 mg.) of *m*-hydroxybenzoic acid, *p*-hydroxybenzoic acid, gentisic acid, γ-resorcylic acid and salicylamide had no effect on glycogen concentration. Salicylic acid, *o*-, *m*- and *p*-cresotic acid and 3-phenylsalicylic acid all possess glycogenolytic activity.

2. A linear relationship was found between the difference in liver-glycogen concentration between corresponding control and treated mice and log dose for salicylic acid.

3. The relative molar potency ratios of *o*-cresotic acid and 3-phenylsalicylic acid as glycogenolytic compounds compared with salicylic acid for the males were *o*-cresotic acid 1·50, 3-phenyl-

salicylic acid 2·68; for the females the respective values were 1·81 and 4·79.

4. The relationship between glycogenolytic and metabolic-stimulant activity is discussed.

REFERENCES

Andrews, M. M. (1958). *Brit. J. Pharmacol.* **13**, 419.
Barbour, H. G. & Devenis, N. M. (1919). *J. Pharmacol.* **13**, 499.
Barnes, J. M. (1953). *Biochem. J.* **54**, 148.
Bartlett, M. S. (1937). *Proc. Roy. Soc.* A, **160**, 268.
Cori, C. F. (1931). *Physiol. Rev.* **11**, 143.
Cramer, W. & Krause, R. A. (1913). *Proc. Roy. Soc.* B, **86**, 550.
Cramer, W. & McCall, R. (1917). *Quart. J. exp. Physiol.* **11**, 59.
Feeney, G. C., Carló, P. & Smith, P. K. (1955). *J. Pharmacol.* **114**, 299.
Fieller, E. C. (1940). *J.R. statist. Soc.* Suppl. **7**, 1.
Finney, D. J. (1952). *Statistical Methods in Biological Assay.* London: C. Griffin and Co.
Good, C. A., Kramer, H. & Somogyi, M. (1933). *J. biol. Chem.* **100**, 485.
Houssay, B. A. (1946). *Vitam. & Horm.* **4**, 187.
Lutwak-Mann, C. (1942). *Biochem. J.* **36**, 706.
May, R. (1909). *Brit. med. J.* **2**, 791.
Meade, B. W. (1954). *Ann. Rheum. Dis.* **13**, 60.
Miller, B. F. & van Slyke, D. D. (1936). *J. biol. Chem.* **114**, 583.
Nissim, J. A. (1953). *Endocrinology*, **52**, 611.
Reid, J. (1957). *Scot. med. J.* **2**, 91.
Singer, H. (1901). *Pflüg. Arch. ges. Physiol.* **74**, 527.
Smith, M. J. H., Meade, B. W. & Bornstein, J. (1952). *Biochem. J.* **51**, 18.
Sproull, D. H. (1954). *Brit. J. Pharmacol.* **9**, 121.
Sutherland, E. W. (1951). *Ann. N.Y. Acad. Sci.* **54**, 693.
Stockman, R. (1912). *J. Pharmacol.* **4**, 98.
Venning, E. H., Kasmin, V. E. & Bell, J. C. (1946). *Endocrinology*, **38**, 79.

Biochem. J. (1960) **75**, 303

The Distribution and Metabolism of Intra- and Extra-Mitochondrial Pyridine Nucleotides in Suspensions of Liver Mitochondria

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The work described in this paper was undertaken to study the penetration of pyridine nucleotides into mitochondria and the interconversion of one nucleotide into another in mitochondrial suspensions. The two methods required for this type of investigation have been developed in this laboratory. They consist of procedures for the rapid separation of mitochondria and medium (Werkheiser & Bartley, 1957) and the determination of the four forms of the pyridine nucleotides as described by Bassham, Birt, Hems & Loening (1959). With these techniques it has been possible to measure the extent of penetration of all four nucleotides into mitochondria at 0° and 25° and to obtain other information about the metabolism of pyridine nucleotides by mitochondria.

MATERIALS

Diphosphopyridine nucleotide (DPN^+) was obtained from Pabst Laboratories, Milwaukee, Wisconsin, U.S.A. It contained 91% of DPN^+ (by the alcohol-dehydrogenase method of Racker, 1950) and no triphosphopyridine nucleotide (TPN^+). Triphosphopyridine nucleotide was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. It contained 92% of TPN^+ when analysed by fluorimetry against a DPN^+ standard solution prepared from the DPN^+ described above. Reduced diphosphopyridine nucleotide (DPNH) was obtained from Sigma Chemical Co. and from C. F. Boehringer und Söhne, Mannheim, Germany. These products contained respectively 90% and 88% of DPNH, when analysed by fluorimetry. Reduced triphosphopyridine nucleotide (TPNH) was obtained from Sigma Chemical Co., and contained 92% of TPNH when analysed by fluorimetry. Adenosine triphosphate (ATP) was obtained as commercial preparations from L. Light and Co. Ltd, Colnbrook, Bucks and Zellstoff-fabrik Waldhof, Pharmazeutische Abteilung, Wiesbaden, Germany.

All reagents used in the analysis of pyridine nucleotides were as described previously (Bassham *et al.* 1959); all other reagents were obtained as commercial products.

Experimental animals. All the rats used were of the white Wistar strain.

METHODS

Preparation of media. For the fractionation of rat liver, a medium containing sucrose (0·25 M) and nicotinamide (25 mM) was prepared in glass-distilled water and was stored at 0–5° before use.

Sucrose media could not be used for the incubation of mitochondria with nucleotides because the hydrolysis products of sucrose formed during the acid extraction of the oxidized nucleotides interfered with the fluorimetric method of analysis. Instead, the medium of Chappell & Perry (1954) was freshly prepared from stock solutions of the components, stored at 0–5°; nicotinamide was added to give a final concentration of 25 mM. The phosphate content of the medium varied somewhat according to the preparation of ATP used. However, it never exceeded 0·7 mM. This concentration is only one-thirtieth of that used by Hunter, Malison, Bridgers, Schutz & Atchison (1959) to deplete mitochondria of their endogenous nucleotides. This medium will be referred to subsequently as the 'saline medium'.

Preparation of mitochondria. The entire tissue fractionation was carried out at 0–2° in an International refrigerated centrifuge, model PR 2. Rat-liver homogenates were prepared in the sucrose medium and the mitochondria isolated by the methods described by Werkheiser & Bartley (1957), with omission of the washing of the first residue. The mitochondrial pellet obtained after centrifuging for 20 min. at 2700 *g* was washed twice by successive resuspensions in 1 vol. of the ice-cold saline medium and centrifuging, first for 10 min. and finally for 5 min., at 8500 *g*. The copious fluffy layer was completely discarded at each decantation. The final mitochondrial residue was dispersed in the saline medium to give a suspension (hereafter 'stock suspension') containing 0·2–0·25 g. wet wt. of mitochondria/ml. The preparation was kept at 0° and the incubation begun as soon as possible.

Incubation. This was carried out in open conical flasks, which were agitated gently during the incubation, either packed in ice (0°) or immersed in water (25°) in a Dubnoff metabolic shaking incubator (Precision Scientific Co., Chicago, Ill., U.S.A.).

A sample (1·9 ml.) of the 'stock suspension' was pipetted into a conical flask immersed in ice and containing a measured volume (0·1 ml.) of saline medium. This suspension ('original mitochondrial suspension') was mixed thoroughly and two samples (0·5 ml.) were pipetted into chilled centrifuge tubes, one of which contained 0·05 ml. of [^{14}C]carboxypolyglucose (labelled polyglucose) (Werkheiser & Bartley, 1957). The mitochondria were packed in each tube by centrifuging at 26 000 *g* for 5 min., the supernatant fluid was decanted into chilled tubes and the pellets were blotted dry with filter paper. The pellet and supernatant fluid containing labelled polyglucose were used to measure the 'acid-insoluble dry matter' (*M*) of the mitochondria, and the extraparticulate fluid volume (*E*) of the pellet, as described by Werkheiser & Bartley (1957) and Amoore (1958). The second sample of supernatant fluid and second pellet were kept in a freezing bath (ethanol–CO_2) for subsequent measurement of the pyridine nucleotides; when the pellet was immersed in the bath, portions (0·2 ml.) of the 'original mitochondrial suspension' were pipetted into the acid and alkaline media for extraction of the nucleotides. Thus the 'original mitochondrial suspension' was allowed to stand at 0° for 8–9 min. before extraction.

As soon as the sampling of the 'original mitochondrial suspension' was complete, a suitable volume of the 'stock suspension' of mitochondria was pipetted into the incubation vessel, in which the appropriate amount of pyridine nucleotide had been dissolved in cold saline medium. The volume of medium was such that the dilution of the 'stock suspension' was the same as that in the 'original mitochondrial suspension'. The contents of the vessel were mixed rapidly and the timing of the incubation was commenced. The entire procedure described above for separation of mitochondrial pellet and supernatant fluid, and sampling of the suspension, was repeated at the appropriate times.

When the incubation was ended, the frozen mitochondrial pellets were resuspended in cold saline medium (2 ml.) and the pyridine nucleotides extracted from 0·5 ml. samples. The temperature of the suspension was kept at 0–2° before extraction. The pyridine nucleotide contents of the supernatant fluids were measured in 0·1 ml. samples.

The procedures described above permitted the measurement of the wet weight and dry weight (*M*) of the mitochondrial pellet, the extraparticulate (*E*) and intramitochondrial fluid volume (*I*) of the pellet, and the pyridine nucleotide content of the whole mitochondrial suspension, isolated mitochondrial pellet and supernatant fluid. The calculation of intramitochondrial nucleotide concentrations was based on the procedure of Werkheiser & Bartley (1957).

Measurement of pyridine nucleotides. DPN^+, DPNH, TPN^+ and TPNH were measured by the fluorimetric method described by Bassham *et al.* (1959). Dilutions of the extracts, when necessary, were made with 0·05 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris)–HCl buffer, pH 8·2.

Measurement of protein content of mitochondria. The acid-insoluble dry matter of the mitochondrial pellets was wet-ashed and the N content of a suitable dilution measured by direct nesslerization. The mitochondrial protein was assumed to contain 16 % of N.

Measurement of chloride. Chloride was measured by the method of Sanderson (1952) as modified by Amoore & Bartley (1958).

RESULTS

Endogenous pyridine nucleotides in mitochondria. The calculation of the values for the endogenous pyridine nucleotide content of mitochondria (Table 1) have been obtained by analysis of resuspended mitochondrial pellets and are based on the 'acid-insoluble dry matter' of the mitochondria. About 66 % of this dry matter is protein, so that values/mg. of protein will be 1½ times as great as those quoted, i.e. total amount of diphosphopyridine nucleotide, 3·8 μmoles/mg. of protein, of

Table 1. *Endogenous pyridine nucleotide content of mitochondria*

Values are means from seventeen different mitochondrial preparations ± S.E.M.

Nucleotide	Amount (μm-moles/mg. dry wt.)
DPN^+	2·02 ± 0·06
DPNH	0·44 ± 0·04
DPN^+ + DPNH	2·46 ± 0·07
TPN^+	1·26 ± 0·10
TPNH	1·08 ± 0·09
TPN^+ + TPNH	2·34 ± 0·12

Table 2. *Oxidation of endogenous pyridine nucleotides by mitochondrial suspensions*

Concentrations listed under the heading Direct were obtained by analysis of the resuspended mitochondrial pellet and those listed under Difference by analysis of the original mitochondrial suspension and of the suspending fluid after sedimentation of the mitochondria. All manipulations were at 0°. For details, see text.

	Concn. of nucleotide in intramitochondrial water (μm-moles/ml.)									
	DPN+		DPNH		TPN+		TPNH		Total	
Expt. no.	Direct	Difference	Direct	Difference	Direct	Difference	Direct	Difference	Direct	Difference
1	1060	840	190	110	850	490	420	530	2520	1970
2	930	510	130	790	670	230	460	920	2190	2440
3	590	340	170	560	430	90	390	780	1560	1770
4	740	750	130	430	490	150	330	950	1690	2280

Table 3. *Changes in the internal water content of mitochondria in the presence of added coenzymes at* 0°

I/M, Internal water (ml.) of the mitochondria/dry wt. (g.) of mitochondria. Original, *I/M* calculated before addition of the nucleotide; (*a*) *I/M* calculated immediately after addition; (*b*) *I/M* calculated 7 or 30 min. after addition. Chloride space, chloride concn. in mitochondrial water/chloride concn. of supernatant fluid expressed as a percentage.

		I/M			Chloride space		
Nucleotide added	Concn. (mM)	Original	(*a*)	(*b*)	Original	(*a*)	(*b*)
DPN+	0·9	2·5	2·5	2·6	92	89	108
	1·6	2·7	2·7	2·8	79	83	83
	7·8	2·4	2·4	2·3	60	65	99
	14·1	2·2	2·2	—	84	108	—
DPNH	0·74	3·1	2·5	2·5	101	86	76
	0·8	2·1	2·3	2·0	77	79	64
	1·85	2·2	2·3	2·2	84	79	82
	6·7	2·3	2·3	2·3	76	74	79
	6·7	2·4	1·7	—	70	44	—
	9·2	2·7	2·0	—	84	80	—
TPN+	0·57	2·4	2·1	2·1	—	—	—
	1·55	3·2	3·1	2·8	100	96	84
	3·8	2·4	2·2	2·3	57	73	73
	11·2	2·2	2·3	—	84	110	—
TPNH	0·85	2·6	2·6	2·5	61	67	66
	1·09	2·5	2·4	2·2	88	82	80
	2·0	2·2	2·4	1·9	100	96	82
	7·15	2·2	1·9	1·8	85	—	75
	8·0	2·4	2·2	—	70	76	—
	12·8	2·7	2·2	—	84	75	—

Table 4. *Changes in the internal water content of mitochondria in the presence of added coenzymes at* 25°

Original, *I/M* calculated for mitochondria before addition of nucleotide, and at 0°; figures in parentheses refer to *I/M* values of these mitochondria after 30 min. incubation at 0°. (*a*) *I/M* immediately after nucleotide addition at 25°; (*b*) *I/M* measured after incubation for 7 min. or 30 min. (*). Chloride space is defined in Table 3.

		I/M			Chloride space		
Nucleotide added	Concn. (mM)	Original	(*a*)	(*b*)	Original	(*a*)	(*b*)
DPN+	0·9	2·5 (2·6)	—	2·5*	92 (89)	—	108*
	7·8	2·7	2·8	2·6	82	75	75
	10·3	3·0	3·0	2·8	88	94	87
TPN+	1·6	3·2 (2·8)	—	2·8*	100 (96)	—	88*
	7·7	2·7	2·9	2·8	82	84	78
	10·4	3·0	2·9	3·0	88	96	108

triphosphopyridine nucleotide, 3·6 μmoles/mg. of protein. The mean value for the ratio internal water content of the mitochondria to dry weight (*I/M*) is about 2·4, hence the original concentration of both di- and tri-phosphopyridine nucleotides in the mitochondrial water is about mM, assuming that all of the nucleotide is in solution. Although the amounts of di- and tri-phosphopyridine nucleotide are approximately equal the variability in the latter is much greater, and more of it is recovered in the reduced form. However, no precise information about the ratios of oxidized and

Table 5. *Total losses of pyridine nucleotides in mitochondrial suspensions*

Values given for percentage concn. are (*a*) for suspensions incubated for periods of either 15 or 30 min. at 0° and (*b*) for suspensions incubated for a further 30 min. at 25°. DPN, Values for DPN^+ plus DPNH; TPN, values for TPN^+ plus TPNH.

Nucleotide added	Initial measured concn. of nucleotides in whole suspension (mM)		Concn. of nucleotides in whole suspension (percentage of initial value)			
			(*a*)		(*b*)	
	DPN	TPN	DPN	TPN	DPN	TPN
DPN^+	0·92	0·036	104	92	61	6
	1·38	0·14	115	107	—	—
	6·80	0·56	111	102	—	—
TPN^+	0·24	0·59	96	105	112	100
	0·52	1·60	98	94	138	75
	1·20	2·70	92	96	—	—
DPNH	0·74	0·20	113	100	—	—
	2·40	0·37	82	130	81	43
	7·80	0·50	91	175	—	—
TPNH	0·32	0·77	122	102	—	—
	0·66	2·0	86	96	119	75
	0·85	6·90	110	107	—	—

Table 6. *Total losses of pyridine nucleotides in supernatant during incubation with mitochondrial suspensions*

DPN, Values for DPN^+ + DPNH; TPN, values for TPN^+ + TPNH.

Nucleotide added	Initial measured concn. of nucleotides in supernatant fluids (mM)		Concn. of nucleotides in supernatant fluids after 15 min. at 25°; (percentage of initial value)	
	DPN	TPN	DPN	TPN
DPN^+	10·3	0	90	0
	7·8	0·11	87	118
TPN^+	4·3	10·4	82	102
	3·0	7·7	50	95

reduced coenzymes in the mitochondria as originally isolated can be obtained from these figures, because the preparation of the pellet for analysis (centrifuging, resuspension for sampling) causes considerable alteration in these ratios. Table 2 presents values obtained at comparable times for DPN^+, DPNH, TPN^+ and TPNH in the mitochondria by analysis of the resuspended pellet and of the original suspension of mitochondria. Generally (e.g. Expts. 2 and 3) there was more oxidized and less reduced coenzyme in the pellet; infrequently (e.g. Expts. 1 and 4) this relationship did not hold. In consequence, the ratios of reduced to oxidized coenzyme (see Table 1) are lower in the mitochondrial pellet than in the original suspension.

Effect of incubation of mitochondria with added nucleotides

Effect on water content of mitochondria. DPN^+ and TPN^+ at the concentrations used produced virtually no change in the proportion of water to dry matter during incubation at either 0° or 25°. DPNH at both higher and lower concentrations sometimes caused a pronounced shrinkage (for example, in one of the quoted experiments at 6·7 mM, I/M decreased by 29% in 15 min.; at 0·74 mM, by 19% in 30 min.). TPNH also caused shrinkage, but the greatest reduction in I/M (18·5% at 12·8 mM) was less than that found with DPNH. It should be noted that in all experiments with TPNH the supernatant fluid contains some DPNH also (e.g. with TPNH 12·8 mM, concn. of DPNH was 0·3 mM).

Effect on the 'chloride space' of mitochondria. At 0°, in the presence of both DPN^+ and TPN^+, the proportion of mitochondrial water penetrated by chloride ('chloride space') usually did not change or increased (Tables 3 and 4); this was true at 25° also, except in one experiment with DPN^+ (7·8 mM) when there was a slight decrease. On the other hand, during the shrinkage produced by DPNH, the 'chloride space' diminished, indicating a selective decrease in the intramitochondrial volume available to chloride. Slight decrease in the 'chloride space' occurred during incubation with TPNH.

Losses of pyridine nucleotides during incubation. The rate of loss of endogenous nucleotides is low (approximately 20% destroyed on incubation for 1 hr. at 25°; Birt, unpublished work), and Table 5 shows that the total coenzyme of mitochondrial suspensions to which either di- or tri-phosphopyridine nucleotides have been added has a similar stability. The greatest loss, in 30 min. at 0°, was 18% of the diphosphopyridine nucleotides when the original concentration of DPNH in the supernatant fluid was 2·4 mM. The rate of destruction was somewhat greater at 25° but analysis of the supernatant fluid of suspensions to which DPN^+ or

Table 7. *Dephosphorylation of triphosphopyridine nucleotide and reduced triphosphopyridine nucleotide by mitochondrial suspensions*

All experiments commenced at 0°. Original percentage composition is that of TPN^+ or TPNH; (*a*) is that immediately (0–2 min.) after addition of nucleotide; (*b*) is that 15–30 min. after addition, still at 0°; (*c*) is that after incubation at 25° from 30 to 60 min. The calculated initial concentration is obtained from the fluorimetric analysis of the solid nucleotides, and the total initial value of added nucleotide, estimated fluorimetrically. Note that this table gives no estimate of the amounts of coenzymes destroyed in the supernatant fluid. Supernatant fluid in experiments with TPN^+ contains only TPN^+ and DPN^+.

		Percentage composition of supernatant fluid							
		TPN				DPN			
Nucleotide added	Calc. initial concn. (mM)	Original	(*a*)	(*b*)	(*c*)	Original	(*a*)	(*b*)	(*c*)
TPN^+	14·8	91	71	—	—	9	29	—	—
	5·0	91	69	69	—	9	31	31	—
	0·68	91	76	76	73	9	24	24	32
TPNH	12·4	92+3*	95+2	—	—	4+1*	2+1	—	—
	7·9	92+3	94+4	—	—	4+1	1+1	—	—
	7·3	92+3	91+2	90+3	—	4+1	6+1	6+1	—
	2·5	92+3	80+0	64+17	8+57	4+1	20+1	12+7	6+29
	1·3	92+3	78+7	59+19	—	4+1	11+4	12+10	—

* Values joined by + are for the reduced and oxidized forms respectively of the di- and tri-phosphopyridine nucleotides.

Table 8. *Dephosphorylation of triphosphopyridine nucleotides by mitochondrial suspensions*

Experiments were done at 25°. Original percentage composition, as described in Table 7; (*a*) as described in Table 7, at 25°; (*b*) 15 min. after addition of TPN^+, at 25°. TPN, Values for $TPN^+ + TPNH$; DPN, values for $DPN^+ + DPNH$.

		Percentage composition of supernatant fluid					
		TPN			DPN		
Nucleotide added	Calc. initial concn. (mM)	Original	(*a*)	(*b*)	Original	(*a*)	(*b*)
TPN^+	13·4	91	71	72	9	29	24
	9·7	91	72	68	9	28	14

TPN^+ were added in concentration above 3 mM showed recoveries greater than 50 % after 15 min. (Table 6).

Dephosphorylation of TPN^+ and TPNH in the supernatant fluid. At 0° there was an initial rapid dephosphorylation of added TPN^+ (Table 7), the proportion dephosphorylated being approximately constant (roughly 30 %) and independent of the original concentration. No further dephosphorylation was observed, except when the original concentration of TPN^+ was low (0·68 mM) and the temperature was raised to 25° for 30 min. When the entire incubation was at 25° and the concentration of TPN^+ high, the same proportion of TPN^+ disappeared in the first few minutes and there was no subsequent dephosphorylation (Table 8).

TPNH was dephosphorylated at a slower rate than TPN^+ (Table 7). The percentage disappearance varied inversely with the original concentration and increased with time and was much faster at 25°. The ultimate products of the reaction were either DPNH or, especially at 25°, DPN^+, but the activity of enzymes destroying these two nucleotides was such that the amount of diphosphopyridine nucleotide appearing was not equal to the amount of triphosphopyridine nucleotide disappearing.

Oxidation of reduced pyridine nucleotides in the supernatant. At 0°, and at all concentrations used, DPNH was oxidized during incubation. The total amount oxidized at 0° was approximately the same, 0·016 μmole/mg. dry wt. in 30 min., independent of the original concentration, and the rate, measured as the percentage oxidized between times *b* and *c* (30 min. at 0°) and *c* and *d* (30 min. at 25°), was increased roughly fivefold by raising the temperature to 25° (Table 9). Oxidation of TPNH (products either TPN^+ or DPN^+) followed the same pattern, but the rates at both 0° and 25° were only one-half to two-thirds of the corresponding values for DPNH.

The oxidation of nucleotides in the supernatant fluid held finally in the mitochondrial pellet was greatly accelerated during the process of centrifuging and resuspension for sampling. The extent of this oxidation can be seen by comparing the amounts of reduced nucleotides in the supernatant fluid in the pellet [calculated from the concentration

of reduced nucleotide in the bulk of the supernatant fluid and the measured volume (E) of extraparticulate fluid] with the total amounts of nucleotide found by direct analysis of the pellet (Table 10). In all experiments with DPNH, the extent of oxidation was so great that the total DPNH of the pellet was much less than that anticipated in the supernatant fluid of the pellet alone, even though all manipulations of the mitochondria before extractions were carried out at 0°. Oxidation could not be prevented, though it was decreased, by heating the pellet at 100° for 2 min. before resuspension.

The rapid oxidation of DPNH sometimes appeared to induce an oxidation of TPNH, so that although the total amount of TPN^+ in the mitochondria was unchanged, a similar discrepancy occurred between TPNH expected and TPNH found in the pellet. Analyses of samples after incubation with TPNH sometimes, though not always, showed the same effect of oxidation in the pellet, though the disagreement between TPNH expected and TPNH found was never so great as in the corresponding experiments with DPNH.

Dephosphorylation of intramitochondrial TPN^+ and TPNH. The extent of this dephosphorylation

Table 9. *Oxidation of added pyridine nucleotides by mitochondrial suspensions in the supernatant fluid*

Initial concentration was calculated as before. (*a*) Percentage oxidized in solid nucleotide added; (*b*) and (*c*), percentage oxidized at 0° after 0–2 min. and after 15 or 30 min. (*) of incubation respectively; (*d*) percentage oxidized after 30–60 min. at 25°. DPN^+ alone appears after addition of DPNH; the values joined by + in the Table for TPNH additions refer to $TPN^+ + DPN^+$.

Added nucleotide	Calc. initial concn. (mM)	Percentage of added nucleotide oxidized in supernatant fluid (*a*)	(*b*)	(*c*)	(*d*)
DPNH	9·0	4	8	—	—
	7·2	4	9	13	—
	2·1	4	13	26*	97
	0·9	4	24	49	—
	0·9	4	18	45	—
TPNH	8·0	3+1	3+2	—	—
	7·2	3+1	2+1	3+1	—
	2·5	3+1	4+2	16+7*	61+3
	1·3	3+1	8+4	28+11	—

Table 10. *Oxidation of added pyridine nucleotides by mitochondrial suspensions in the pellet*

Mitochondrial suspension at the temperature shown was sampled at various times; analytical figures given are for the whole pellet and figures (E) are calculated for the measured volume of supernatant fluid entrapped in the pellet.

Added nucleotide	Calc. initial concn. (mM)	Temp. of suspension	Time of incubation (min.)	Source of pyridine nucleotide	Amount of coenzyme (μm-moles) DPN^+	DPNH	TPN^+	TPNH
DPNH	7·3	0°	0	Pellet	640	65	92	37
				E	38	540	1	53
	7·3	0	15	Pellet	640	79	73	76
				E	59	550	2	57
	8·0	0	0	Pellet	410	104	68	60
				E	42	410	1	92
	2·1	0	0	Pellet	150	28	45	28
				E	12	56	0	8
	2·1	25	60	Pellet	160	21	55	24
				E	62	5	1	6
TPNH	7·9	0	0	Pellet	88	170	96	370
				E	6	7	15	410
	7·2	0	15	Pellet	68	73	105	370
				E	4	18	85	70
	2·4	0	0	Pellet	120	71	140	170
				E	4	33	6	120
		25	60	Pellet	130	24	180	20
				E	29	6	64	8

Table 11. *Changes in intramitochondrial pyridine nucleotides on addition of di- or tri-phosphopyridine nucleotide*

Expts. 5 and 6 were done with one preparation of mitochondria, incubated with the nucleotides at 25°. All other experiments were at 0°. (*a*) Before addition of nucleotide; (*b*) immediately after addition; (*c*) 15 or 30 min. after addition.

Expt. no.	Nucleotide added	Nucleotide measured	Concn. in supernatant fluid (mM)			Amount in mitochondria (μm-moles/mg. dry wt.)		
			(*a*)	(*b*)	(*c*)	(*a*)	(*b*)	(*c*)
1	DPN+	DPN+	0	1·6	1·5	1·70	1·60	2·40
		DPNH	0	0	0	0·56	0·68	0·47
		TPN+	0	0·090	0·10	0·87	0·88	1·20
		TPNH	0	0	0	1·58	1·30	1·10
2	DPN+	DPN+	2	7·8	7·9	2·50	5·20	6·70
		DPNH	0	0·008	0·004	0·34	0·48	0·34
		TPN+	1	0	0	1·30	1·90	1·90
		TPNH	0	0·016	0·008	1·20	0·96	0·23
3	TPN+	DPN+	3	0·350	0·380	1·60	2·00	2·50
		DPNH	0	0	0	0·40	1·40	2·30
		TPN+	0	1·6	1·600	1·20	0·73	0·38
		TPNH	0	0	0	0·64	1·40	1·40
4	TPN+	DPN+	8	1·7	1·7	2·30	2·70	2·70
		DPNH	0	0	0	0·15	0·27	0·25
		TPN+	2	3·8	3·8	0·75	5·20	4·90
		TPNH	0	0	0	0·86	0·74	1·00
5	DPN+	DPN+	0	7·8	6·8	2·0	5·70	8·8
		DPNH	0	0	0	0·48	0·17	0
		TPN+	0	0·11	0·13	1·20	1·80	2·90
		TPNH	0	0	0	1·70	0·50	0·00
6	TPN+	DPN+	0	3·0	1·5	2·0	0·00	2·60
		DPNH	0	0	0	0·48	0·90	0·87
		TPN+	0	7·7	7·3	1·20	11·7	16·3
		TPNH	0	0	0	1·70	1·20	1·30

Table 12. *Changes in intramitochondrial nucleotides on addition of reduced di-or tri-phosphopyridine nucleotide*

All experiments were at 0°. (*a*) Before addition of nucleotide; (*b*) immediately after addition; (*c*) 15 or 30 min. after addition. Where values for DPN+ + DPNH and TPN+ + TPNH are bracketed, oxidation in the pellet prevented separate estimation of the oxidized and reduced forms.

Expt. no.	Nucleotide added	Nucleotide measured	Concn. in supernatant fluid (mM)			Amount in mitochondria (μm-moles/mg. dry wt.)		
			(*a*)	(*b*)	(*c*)	(*a*)	(*b*)	(*c*)
1	DPNH	DPN+	0·007	0·270	0·540	1·90	4·2	5·7
		DPNH	0	1·85	1·20	0·55		
		TPN+	0·003	0·005	0·12	1·50	3·2	3·0
		TPNH	0	0·180	0·29	1·40		
2	DPNH	DPN+	0·005	0·58	0·94	2·40	5·8	6·5
		DPNH	0	6·70	6·40	0·44		
		TPN+	0·005	0·06	0·06	1·90	2·5	3·1
		TPNH	0	0·66	0·72	0·97		
3	TPNH	DPN+	0·005	0·05	0·10	2·00	2·4	2·8
		DPNH	0	0·09	0·14	0·67	2·0	1·20
		TPN+	0·003	0·13	0·33	0·85	1·8	1·8
		TPNH	0	0·85	0·53	1·30	0·67	1·4
4	TPNH	DPN+	0·006	—	0·09	1·80	—	2·4
		DPNH	0	—	0·44	0·46	—	2·1
		TPN+	0·006	—	0·20	1·30	—	3·6
		TPNH	0	—	7·10	1·40	—	2·6
5	TPNH	DPN+	0·01	—	0·13	2·10	—	9·3
		DPNH	0	—	0·14	0·87		
		TPN+	0	—	0·29	1·30	—	1·8
		TPNH	0	—	8·0	0·98		

varied, but in every experiment with TPN^+ and TPNH there was an increase in the total amount of diphosphopyridine nucleotide in the mitochondria (Table 11, Expts. 3, 4, 6; Table 12, Expts. 3, 4, 5). Sometimes there was also an increase in the TPN^+ (Table 11, Expts. 3, 6; Table 12, Expt. 4). The temperature of incubation did not have a marked influence on the rate of dephosphorylation (compare Table 11, Expt. 6; Table 12, Expt. 5).

Oxidation of intramitochondrial nucleotides. The occurrence of a rapid oxidation in the packed mitochondrial pellet of endogenous (Table 2) and added (Table 10) reduced nucleotides has already been discussed. Similarly, DPNH and TPNH entering the mitochondria were readily oxidized (Table 12). This oxidation always prevented the measurement of the levels of individual intramitochondrial coenzymes (DPN^+ and DPNH, and sometimes TPN^+ and TPNH) after incubation with DPNH, and occasionally after incubation with TPNH. The rapid oxidation of TPNH in one experiment (Table 12, Expt. 5) was accompanied by considerable dephosphorylation. However, it is always possible to calculate the increases in total di- and tri-phosphopyridine nucleotides of the mitochondria.

Changes in amounts of intramitochondrial nucleotides. During incubation with DPN^+, at both higher and lower concentrations, there was an increase in the amount of DPN^+ in the mitochondria, no change in TPN^+ and a decrease in both DPNH and TPNH. These changes were found at 0° and at 25°, when the loss of reduced pyridine nucleotide was accelerated.

Incubation with TPN^+ always increased the amount of DPN^+ in the mitochondria, and sometimes that of the TPN^+. At 0°, DPNH and TPNH were either maintained or increased during incubation; at 25°, the decline in the reduced nucleotides was less rapid than with added DPN^+.

The amount of DPN^+ always increased during incubation with either DPNH or TPNH; DPNH did not affect the level of TPN^+, whereas TPNH in some experiments increased it.

Permeability to added pyridine nucleotides

Table 13 presents values for the permeability of different mitochondrial preparations to DPN^+, DPNH, TPN^+ and TPNH, each at three different concentrations. Permeability has been calculated as the ratio of the total increase in the concentration of intramitochondrial pyridine nucleotides to the total increase in the concentration of extramitochondrial nucleotides, expressed as a percentage. The choice of total values as giving the most useful measure of permeability was determined by the transformations which both extra- and intramitochondrial coenzymes undergo during incubation. All the values for increases in concentration have been corrected for changes in the water content/unit dry weight of the mitochondria, so that swelling and shrinking, which were usually slight, do not influence the results obtained.

Comparison of the results obtained with DPN^+ and TPN^+ shows that at each concentration, except the lowest, the permeability to TPN^+ was greater; in the exceptional experiment, the external concentration of TPN^+ was lower than the initial internal concentration. If the permeabilities are calculated from the external concentration of the particular nucleotide being investigated (i.e. either DPN^+ or TPN^+) rather than the total external concentration, the relative permeability

Table 13. *Permeability of mitochondria to nucleotides*

Permeability, total increase in concentration of intramitochondrial pyridine nucleotides/total increase in concentration of extramitochondrial nucleotides, expressed as percentage. All values have been corrected for swelling or shrinking of the mitochondria during incubation. (*a*) Permeability immediately after addition of nucleotide at 0°; (*b*) permeability 15 or 30 min. after addition of nucleotide at 0°; (*c*) permeability after incubation for 30–60 min. at 25°.

Nucleotide added	Expt. no.	Concn. of added nucleotide (total) (mM)			Permeability (%)		
		(*a*)	(*b*)	(*c*)	(*a*)	(*b*)	(*c*)
DPN^+	1	0·90	0·91	0·60	22	12	16
	2	1·7	1·6	—	0	6	—
	3	7·8	7·9	—	17	23	—
TPN^+	4	0·75	0·75	0·80	0	102	6
	5	1·9	2·0	2·00	28	53	37
	6	5·5	5·5	—	40	38	—
DPNH	7	1·1	1·0	—	61	65	—
	8	2·3	2·2	2·4	37	71	100
	9	8·0	8·1	—	13	21	—
TPNH	10	1·1	1·1	—	74	86	—
	11	2·7	2·5	2·6	91	110	77
	12	—	7·8	—	—	44	—

to TPN is even greater. For example, permeabilities calculated in this way immediately after the addition of the nucleotides are (*a*) for DPN^+ unchanged (Table 13, Expts. 2 and 3) and (*b*) for TPN^+ increased from 28 to 34% (Table 13, Expt. 5) and from 40 to 58% (Table 13, Expt. 6). Thus the correct figures for permeability to TPN^+ will always be somewhat higher than those shown in Table 13, whereas the correct values for DPN^+ will be almost identical with those of the table.

Permeability to TPNH is greater than that to DPNH at comparable concentrations. Application of corrections for dephosphorylation increased the value for permeability to TPNH slightly, but not as greatly as for TPN^+, because of the lower rate of dephosphorylation of TPNH. Similarly, the calculated value of the permeability to DPNH will be increased by using the external concentration of DPNH alone, but the increase is not sufficient to abolish the difference between the permeabilities to the two nucleotides. For example, the corrected permeability to DPNH (see Table 13, Expt. 9) is 26% instead of 21% after 15 min. at 0°; to TPNH (Table 13, Expt. 12) it is 48% instead of 44%.

The permeability to all four nucleotides generally increased on incubation at 0°, and did not change markedly when the temperature was raised to 25°.

There was no evidence for the adsorption of DPN^+, TPN^+ or TPNH, but the permeability (as previously defined) to DPNH increased as the external concentration decreased, suggesting considerable adsorption of this coenzyme. Therefore direct comparisons between the permeabilities of di- and tri-phosphopyridine nucleotides were made by incubating different samples of the same mitochondrial suspension with high concentrations of DPN^+ and TPN^+, and DPNH and TPNH. These experiments (Table 14) confirmed the results already described (Table 13) at 0°; further, the same marked difference was demonstrated at 25°, at which temperature the mitochondria are capable of metabolic activities.

Effect of change in mitochondria on permeability to nucleotides. The demonstration of the difference in permeability to di- and tri-phosphopyridine nucleotides was dependent on the condition of the mitochondria in the original suspension. Thus increase in the original value of I/M, decrease in the initial proportion of reduced nucleotide (especially DPNH) and loss of ability to maintain reduced nucleotides during incubation, all of which can be considered as degenerative changes, were observed to accompany a considerable increase in the permeability to DPN^+ and DPNH, whereas that to TPN^+ and TPNH did not change (Table 15). This alteration in relative permeability was found only when all of

Table 14. *Permeability of mitochondria to pyridine nucleotides: direct comparisons*

Permeability is defined in Table 13. All values are corrected for swelling or shrinking of the mitochondria during incubation. (*a*) Values immediately after addition of nucleotide; (*b*) values after 15 min.

Expt. no.	Nucleotide added	Temp. of incubation	Concn. of added nucleotide (total) (mM) (*a*)	(*b*)	Permeability (%) (*a*)	(*b*)
1	DPN^+	0°	14	—	25	—
	TPN^+		16	—	41	—
2	DPNH	0	8·8	—	0	—
	TPNH		8·5	—	37	—
3	DPN^+	25	7·9	6·9	12	28
	TPN^+		11	8·8	30	60

Table 15. *Comparison of permeability of different preparations of mitochondria*

Mitochondria in Expts. 2 and 4 had undergone the degenerative changes described in the text. Expts. 1 and 2, incubation at 0°; Expts. 3 and 4, incubation at 25°. Permeability was measured immediately after addition of the nucleotides. DPN and TPN refer to total di- and tri-phosphopyridine nucleotides respectively.

	Mitochondria before addition of nucleotide			Percentage of original total reduced nucleotide retained on incubation with				Permeability (%)	
		Percentage of nucleotide reduced		DPN		TPN			
Expt. no.	I/M	DPN	TPN	2 min.	15 min.	2 min.	15 min.	DPN^+(H)	TPN^+(H)
1 (DPNH, TPNH)	2·4	42	42	—	—	—	—	0	37
2 (DPNH, TPNH)	2·7	8	69	—	—	—	—	47	40
3 (DPN^+, TPN^+)	2·7	25	59	30	0	62	64	12	28
4 (DPN^+, TPN^+)	2·95	13	44	0	0	88	0	39	27

the three variations in the original mitochondrial suspension had occurred and never when only one was detected.

DISCUSSION

Description of some properties of mitochondria prepared in saline medium. The initial water content (I/M mean 2·4 approximately) of mitochondria in the saline medium was higher than those of mitochondria suspended in sucrose (e.g. 1·6–1·9, Werkheiser & Bartley, 1957), and the proportion of dry matter in the mitochondria is therefore somewhat lower (approximately 30 %).

The 'chloride space' (range 63–100 %) of the original mitochondrial suspension in saline medium was usually larger after comparable times of incubation than that of mitochondria in sucrose plus chloride (34–74 %; Amoore & Bartley, 1958), indicating that there had been an increase in the permeability to chloride after transfer from sucrose.

Endogenous pyridine nucleotides in mitochondria. Published values for the levels of the coenzymes of rat-liver mitochondria show considerable variation. Table 16 summarizes recent work.

With the mitochondria used in this investigation (66 % protein) the total level of pyridine nucleotide/mg. of protein was 7·3 μmoles (Table 16), a value close to those of Jacobson & Kaplan (1957*a*) and Chance & Williams (1955). The figures given by Glock & McLean (1956) and Maley & Lardy (1955) are much lower, as are the initial levels quoted by Purvis (1958). The values in parentheses in Table 16 are for the levels of di- and tri-phosphopyridine nucleotide after release of the 'inhibited forms' of these coenzymes (Purvis, 1958). If the distinction drawn by Purvis between 'inhibited' and free forms of DPN and TPN is applicable to the nucleotides of the mitochondria in the saline medium, it would seem that the extraction procedure used removes both forms of the coenzymes. Thus the total amount of pyridine nucleotide present is measured, irrespective of its intramitochondrial location [compare the evidence presented by Lehninger, Wadkins, Cooper, Devlin & Gamble (1958) and Chance & Baltscheffsky (1958) for the binding of coenzymes to particular dehydrogenase systems].

Glock & McLean (1956) and Purvis (1958) find approximately equal amounts of di- and triphosphopyridine nucleotides in liver mitochondria, as described also in this paper; whereas Jacobson & Kaplan (1957*a*) report a considerable excess of TPN^+.

In all the analyses quoted the ratio $TPNH/TPN^+$ is much greater than the ratio $DPNH/DPN^+$. However, these ratios are of doubtful significance, as they may reflect only the conditions in which the mitochondria were isolated (see Dickens, Glock & McLean, 1959). It would appear that the oxidation of endogenous nucleotides occurring when the mitochondria are sedimented during the isolation procedure (as in the experiments described in Table 2) will result in decrease in the ratio of reduced to oxidized coenzymes, if no reversal of the process occurs during resuspension.

Changes in mitochondrial properties after addition of pyridine nucleotides. The shrinkage of mitochondria with DPNH and TPNH is probably due to a complex series of reactions, especially in view of the recent work of Lehninger, Ray & Schneider (1959), which ascribes a particular role to DPNH in the maintenance of the structure of the mitochondrial membrane. It is possible that the shrinkage is mediated by a transhydrogenation to the endogenous nucleotides in the presence of added DPNH and TPNH; however, as the hydrogen

Table 16. *Concentrations of endogenous pyridine nucleotides in mitochondria*

Authors	Concn. (μm-moles/mg. of protein) DPN	TPN	Total pyridine nucleotide	Remarks
(1) Maley & Lardy (1955)	—	—	Approx. 2·9	Calculated from value for total pyridine nucleotide/mg. of mitochondrial N
(2) Chance & Williams (1955)	—	—	Approx. 7·1	Calculated from difference spectrum for anaerobic mitochondria and protein content of mitochondrial suspension
(3) Glock & McLean (1956)	1·9	1·8	3·7	Calculated from pyridine nucleotide and N values given for unwashed mitochondria
(4) Jacobson & Kaplan (1957*a*)	2·0	4·3	6·3	Calculated from values given as g./mg. of protein
(5) Purvis (1958)	2·7 (4·3)	2·4 (4·9)	5·1 (9·2)	Quoted directly
(6) This paper (1960)	3·7	3·6	7·3	—

transfer to the endogenous nucleotides is more marked with TPNH than DPNH, whereas the shrinkage is less so, it seems more likely that the fall in values of I/M is due specifically to the addition of DPNH itself. The relatively smaller shrinkage found with TPNH may be due to its partial transformation into DPNH. The decrease in 'chloride space' produced by DPNH (and TPNH) is further evidence for a profound change in the structural organization of the mitochondria exposed to these two nucleotides.

Metabolism of pyridine nucleotides added to mitochondrial suspensions. Jacobson & Kaplan (1957*b*) found that rat-liver mitochondria contain DPN^+ and DPNH pyrophosphatases of low activities (approximately one-tenth of that of the microsomes) but were unable to detect DPN-nucleosidase activity. Sung & Williams (1952) report slight DPN-nucleosidase activity. Our results are consistent with these findings. Added DPN^+, DPNH and TPNH disappeared slowly during incubation, at rates comparable with those for the loss of endogenous coenzymes, but TPN^+ was rapidly dephosphorylated in the early stages of the incubations (compare the dephosphorylation of added ATP by rat-liver mitochondria at 0° described by Amoore, 1958). This may be due to the action of the non-specific phosphatases of either the mitochondria (e.g. alkaline phosphatase; Allard, de Lamirande, Faria & Cantero, 1954) or contaminants derived from the soluble part of the cell. The latter seems likely to account for at least some of the dephosphorylation, as the endogenous TPN^+ is much more stable. The rate of dephosphorylation was far greater initially but the small losses occurring during incubation with low concentrations of TPN^+ at 25° suggest that the process does continue at a much reduced rate.

The more rapid oxidation in the supernatant fluid of added DPNH, compared with that of added TPNH, agrees with the findings of Joshi, Newburgh & Cheldelin (1957) and Baessler & Pressman (1959). The absolute rate of oxidation was low: in the first 30 min. of incubation at 0° with 2·1 mM-DPNH the rate of consumption of oxygen required to account for the measured disappearance of DPNH was 0·18 μl./mg. dry wt./hr., rising to 1·0 μl./mg. dry wt./hr. when the temperature was raised to 25°. (These values may be compared with the rate of oxygen consumption required to account for the loss of endogenous reduced nucleotides when mitochondria were incubated at 25° without substrate: approximately 0·13 μl./mg. dry wt./hr.) Oxidation (assessed by the loss of DPNH balanced by a gain in DPN^+) occurred even when the permeability of the mitochondria was extremely low, perhaps by a process akin to the non-phosphorylating pathway of Lehninger (1955–56). He has reported that mitochondria in sucrose will oxidize DPNH readily only when they have been pretreated in such a way as to increase their permeability; but Maley (1957), who incubated mitochondria with DPNH (and ethanol) in the medium, found a much more vigorous oxidation without pretreatment. This worker notes, however, that the presence of the ethanol may alter the permeability to nucleotides. The great increase in the rate of oxidation of reduced nucleotides which occurs when the mitochondria are packed during centrifuging was surprising, as it was thought that the relatively anaerobic conditions would promote further reduction. It is known, however, that both DPNH and TPNH are oxidized non-enzymically in solutions of free flavins (Singer & Kearney, 1950; Bassham *et al.* 1959), and the oxidation in the pellets is at least partly non-enzymic, as it cannot be prevented by heating. During the packing of the pellet, the concentrations of compounds (including flavins) released from damaged particles will increase greatly, and the rate of oxidation is accelerated correspondingly. This cannot, however, be a valid explanation for the greater rate of oxidation of the endogenous nucleotides, which are retained in the mitochondria during centrifuging.

It has been shown that a number of transformations of added pyridine nucleotides proceed in the supernatant fluid of the incubated mitochondrial suspension. These must result from the release of catalytic compounds (e.g. flavins or enzymes) from the particulate material, or occur at the surface of the mitochondria.

Permeability of mitochondria to nucleotides. Because of the complexity of the transformation which the nucleotides undergo during incubation with mitochondria it is convenient to summarize the information obtained by a diagram (Fig. 1). This illustrates all the reactions which have been observed during incubations with all four coenzymes; in any particular experiments with a given nucleotide, at least some, but usually not all, of the possible transformations of the nucleotide were seen. Mitochondria have a low permeability to both DPN^+ and DPNH. DPNH is strongly adsorbed and is oxidized in the mitochondria. DPN^+ that enters the mitochondria does not appear to influence the behaviour of the endogenous reduced nucleotides, and is never itself reduced. Thus it apparently does not enter into the 'pool' of functional endogenous coenzymes. It is not possible to draw any analogous conclusions about the behaviour of DPNH entering the mitochondria, but the permeability to this nucleotide was usually lower than to DPN^+.

Permeability to the diphosphopyridine nucleotides may be increased by altering the properties of the mitochondria used (see also Lehninger, 1951),

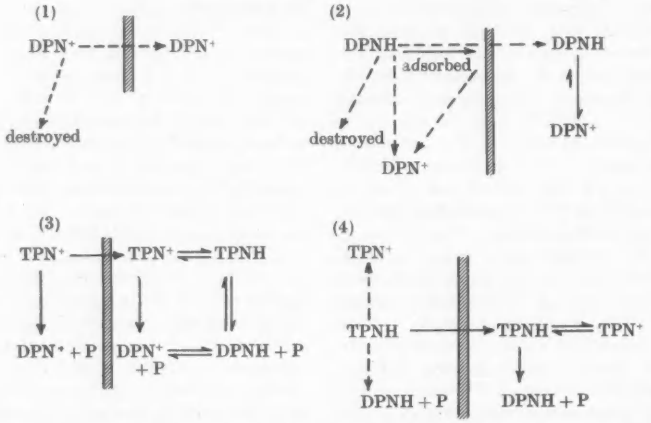

Fig. 1. Transformations of pyridine nucleotides incubated with mitochondria. Broken lines indicate slow reactions, continuous lines rapid reactions. The hatched area represents the mitochondrial membrane, the areas to the left and right of it the extra- and intra-mitochondrial environments respectively. Reactions which have been described for a particular nucleotide in (1) are not repeated in (2), (3) or (4), in which the nucleotide appears. P indicates the liberation of orthophosphate.

and was more than doubled in preparations with higher I/M, lower original level of reduced pyridine nucleotide and decreased ability to maintain reduced nucleotides. These changes are consistent with swelling and disruption of the mitochondrial structure, processes which, when induced by pretreatment with phosphate, produce mitochondrial suspensions which have lost most of their endogenous nucleotides but have the ability to incorporate large quantities of DPN^+ (Hunter *et al.* 1959). The final concentration of DPN in these mitochondria may be much greater than the original concentration, and at least some of the reincorporated nucleotide is bound in such a way as to function in oxidative phosphorylation. In our experiments, the degree of disorganization of the mitochondria and the loss of endogenous nucleotides during preparation (estimated by comparing the initial amounts of the coenzymes with the values of Table 1), though much less, were sufficient to permit a relatively great uptake of DPN^+ and DPNH.

Both TPN^+ and TPNH enter the mitochondria readily. TPN^+ increases or maintains the level of reduced nucleotides (TPNH and DPNH) at both 0° and 25°, and so appears to have access to sites involved in intramitochondrial oxidation and reduction. However, as the rate of dephosphorylation of TPN^+ entering the mitochondria was much higher than that of the endogenous TPN^+, it is also apparent that the intramitochondrial location of much of the TPN^+ is distinct from that of the endogenous TPN^+. It is not possible to make similar observations about TPNH.

The experiments described in this paper give direct experimental support to the notion that isolated undamaged mitochondria can exclude added DPN^+ and DPNH from participation in intramitochondrial processes of oxidation and reduction. Some binding of the nucleotide and alteration of mitochondrial structure may occur with DPNH. TPN^+ and TPNH, on the other hand, can enter the mitochondria readily and participate in oxidations and reductions. Thus it seems that there is a possibility of the passage of hydrogen, available either for energy production via transhydrogenation in the mitochondria or for reductive syntheses, across the mitochondrial membrane.

SUMMARY

1. The distribution of added and endogenous pyridine nucleotides between rat-liver mitochondria and the medium in which they were suspended has been studied. The distribution of the added coenzymes between the suspending fluid and the mitochondria has been interpreted in terms of the permeability of the mitochondria to the pyridine nucleotides.

2. The amounts of endogenous nucleotides (as μmoles/mg. dry wt.) were: DPN^+ 2·02; DPNH 0·44; TPN^+ 1·26; TPNH 1·08. Of the dry matter of the mitochondria, 66% was protein. Considerable oxidation of the reduced nucleotides occurred when the particles were sedimented.

3. Addition of DPNH and TPNH sometimes produced a shrinkage of the mitochondria, the

maximum decrease in the proportion of water to dry matter in the particles being 29% in 15 min. with DPNH (6·7 mM) and 18·5% with TPNH (12·8 mM). During shrinkage, there was a selective decrease in the 'chloride space' of the mitochondria.

4. Loss of added nucleotides was always slight at 0°, the greatest amounting to 18% disappearance of DPNH (originally 2·4 mM) in 30 min. The maximum rate of loss was approximately trebled when the temperature was raised to 25°.

5. TPN^+ and TPNH were dephosphorylated, the former more rapidly; the products appeared in both the mitochondria and supernatant fluid.

6. DPNH was oxidized slowly (approximately 0·032 μmole/mg. dry wt./hr.) in the suspension, but much more rapidly when the mitochondria were sedimented. The rate of oxidation of TPNH under similar conditions was approximately two-thirds of the corresponding values for DPNH.

7. After addition of DPN^+, DPNH, TPN^+ and TPNH at concentrations greater than 5 mM to different suspensions, the mitochondria contained DPN^+ and DPNH at concentrations 0–25% and TPN^+ and TPNH at concentrations 37–41% of that in the supernatant fluid. This difference in 'permeability' was demonstrated over a wide range (2–15 mM) of concentrations and at both 0° and 25°. Only DPNH was adsorbed by the mitochondria.

8. TPN^+ and TPNH permeating the mitochondria enter the 'pool' of functional nucleotides.

9. Degenerative changes in the original mitochondrial preparations (swelling, low level of endogenous reduced nucleotides and inability to maintain reduced nucleotides) resulted in a greatly increased 'permeability' to the diphosphopyridine nucleotides.

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REFERENCES

Allard, C., de Lamirande, G., Faria, H. & Cantero, A. (1954). *Canad. J. Biochem. Physiol.* **32**, 383.
Amoore, J. E. (1958). *Biochem. J.* **70**, 718.
Amoore, J. E. & Bartley, W. (1958). *Biochem. J.* **69**, 223.
Baessler, K. H. & Pressman, B. C. (1959). *Fed. Proc.* **18**, 184.
Bassham, J. A., Birt, L. M., Hems, R. & Loening, U. L. (1959). *Biochem. J.* **73**, 491.
Chance, B. & Baltscheffsky, H. (1958). *J. biol. Chem.* **233**, 736.
Chance, B. & Williams, G. R. (1955). *J. biol. Chem.* **217**, 395.
Chappell, J. B. & Perry, S. V. (1954). *Nature, Lond.*, **173**, 1094.
Dickens, F., Glock, G. E. & McLean, P. (1959). *Ciba Foundation Symp., Regulation of Cell Metabolism*, p. 183. Ed. by Wolstenholme, G. E. W. & O'Connor, C. M. London: J. and A. Churchill Ltd.
Glock, G. E. & McLean, P. (1956). *Exp. Cell Res.* **11**, 234.
Hunter, F. E., Malison, R., Bridgers, W. F., Schutz, B. & Atchison, A. (1959). *J. biol. Chem.* **234**, 693.
Jacobson, K. B. & Kaplan, N. O. (1957*a*). *J. biol. Chem.* **226**, 603.
Jacobson, K. B. & Kaplan, N. O. (1957*b*). *J. biophys. biochem. Cytol.* **3**, 31.
Joshi, S., Newburgh, R. W. & Cheldelin, V. H. (1957). *J. biol. Chem.* **229**, 771.
Lehninger, A. L. (1951). *J. biol. Chem.* **190**, 345.
Lehninger, A. L. (1955–56). *Harvey Lect.* **49**, 176.
Lehninger, A. L., Ray, B. L. & Schneider, M. (1959). *J. biophys. biochem. Cytol.* **5**, 97.
Lehninger, A. L., Wadkins, C. L., Cooper, C., Devlin, T. M. & Gamble, J. L. (1958). *Science*, **128**, 450.
Maley, G. F. (1957). *J. biol. Chem.* **224**, 1029.
Maley, G. F. & Lardy, H. A. (1955). *J. biol. Chem.* **215**, 377.
Purvis, J. L. (1958). *Nature, Lond.*, **182**, 711.
Racker, E. (1950). *J. biol. Chem.* **184**, 313.
Sanderson, P. H. (1952). *Biochem. J.* **52**, 502.
Singer, T. P. & Kearney, E. B. (1950). *J. biol. Chem.* **183**, 409.
Sung, S. & Williams, J. N. (1952). *J. biol. Chem.* **197**, 175.
Werkheiser, W. C. & Bartley, W. (1957). *Biochem. J.* **66**, 79.

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Lipid Synthesis by Human Leucocytes *in vitro*

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Synthesis of lipid by human and rabbit erythrocytes *in vitro* has been reported by several investigators (Altman, Watman & Salomon, 1951; Altman 1953; James, Lovelock & Webb, 1957; Marks, Johnson, Hirschberg & Banks, 1958; Rowe, 1959; Turner, 1958). In all these studies blood, sometimes with the 'buffy coat' removed after centrifuging, has been incubated with [^{14}C]acetate. After incubation the cells, plasma or whole blood have been extracted for lipids and this lipid extract

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or its fractions has been measured for radioactivity. The erythrocyte lipids are labile and readily removed by washing (Lovelock, 1955), and they appear to decrease as the cell ages (Prankerd, 1958).

The erythrocyte lipid is mainly structural in function (Ponder, 1954) and it is thought that the function of the lipid synthesis is to maintain the integrity of the erythrocyte membrane. Marks *et al.* (1958) have shown that, if erythrocytes are centrifuged, the top young cells have a greater ability to synthesize lipid than the bottom older cells.

During an attempt to measure quantitatively the rate of lipid synthesis by blood cells, it became apparent that duplicate readings on the same blood samples gave very different rates of incorporation of radioactivity from [^{14}C]acetate into the lipid fraction. In agreement with Marks *et al.* (1958) it was observed that, after centrifuging, the top layers of cells gave considerably higher readings than the bottom layers of cells, the bottom cells on some occasions showing complete inactivity.

Since removal of the 'buffy' layer after centrifuging removes only 60–80% of the leucocytes (McKane & Ingram, 1957), leaving the remaining leucocytes mainly in the upper layers of the erythrocytes (Burt & Rossiter, 1950), and since lipid synthesis by mature erythrocytes seems unlikely in view of their lack of respiratory enzymes (Granick, 1949) or lipase (Valentine, 1958), it was thought that a possible explanation of the lack of reproducibility of results could be that the lipid synthesis is performed mainly or entirely by the white cells.

METHODS

General conditions. All blood samples were taken from normal healthy volunteers. Glassware and syringes were treated with silicone and heparin or ethylenediaminetetraacetic acid or both were used as anticoagulants. Whether heparin or ethylenediaminetetra-acetic acid was used made no difference to the experimental results. Blood samples were kept chilled during all manipulations until incubated. All cells were incubated in a measured sample of their own plasma (previously obtained by centrifuging) at 37°, being gently agitated throughout.

Preparation of leucocytes. Leucocytes were prepared by dextran sedimentation (Davis, Spun & Wilson, 1958), centrifuging (Jago, 1956; McKane & Ingram, 1957) or by haemolysis of erythrocytes with saponin (Schwartz, Grazia, Cheney, Hasenfus & Cohen, 1956). By the last named method the total leucocytes from 1 ml. samples of blood were obtained, the erythrocyte haemolysis being performed with the minimum concentration of saponin necessary in cold aqueous 0·6% NaCl. Control total cell samples from similar 1 ml. samples of the same blood were washed with the same concentration of saponin in cold aqueous 0·9% NaCl (this does not haemolyse the erythrocytes).

Preparation of erythrocytes. Erythrocytes have not been obtained completely free from leucocytes, but the leucocyte count can be reduced to less than 50/mm.^{3} (total packed cell volume 50%) by filtration of the blood through cotton wool (Fleming, 1926) and repeatedly centrifuging and discarding the upper layers of cells (Munn, 1958).

By these methods reticulocytes and very young cells are discarded, but it has been found impossible to separate reticulocytes from leucocytes.

Labelling and measurement of radioactivity. Sodium [^{14}C]acetate (68 μc/mg.), approx. 1 μc/ml., or [^{32}P]orthophosphate (>1 c/mg.), 100 μc/ml., was added to the cells in plasma just before incubation, the exact number of counts added being determined by counting a measured sample of the plasma immediately after mixing. All counting was at infinite thickness with a thin end-window counter (1·7 mg./cm.^{2}), counting being done for a sufficient time to give an accuracy of ± 4% in all except the least-active samples.

Separation of lipids. After incubation cells were centrifuged and washed once with aqueous 0·9% NaCl. The total lipid was extracted from the cells and the plasma by the method of Folch, Lees & Sloane-Stanley (1957); the extract was evaporated to small volume under reduced pressure and transferred to planchettes. The lipid was weighed on the planchettes.

RESULTS

All radioactivity has been expressed as counts/mg. of lipid extracted/min. and corrected to 10 000 counts/ml./min. added to the plasma before incubation.

The amounts of lipid extracted from plasma and erythrocytes agreed very well with reports of previous investigators [erythrocytes approx. 5 mg./ml. of cells (Wintrobe, 1956); plasma approx. 8 mg./ml. (Peters & van Slyke, 1946)]. The leucocyte lipid was found to be approx. 19 mg./ml., slightly higher than that reported by Boyd (1936) but agreeing with the value given by Burt & Rossiter (1950).

It was found that in all cases leucocytes were 100–1000 times more active in the uptake of radioactivity into their lipid fraction from the labelled acetate than were erythrocytes. In a few experiments where erythrocytes have been obtained uncontaminated by leucocytes (less than 50 mm.^{3}), the erythrocytes have been found to be completely inactive.

Figs. 1 and 2 show the results of typical experiments. The labelled lipid was found both in the cells and the plasma in which the cells were incubated. The rate of labelling of the lipid was initially extremely rapid, the labelling being detectable after 2 min. in both the cells and plasma (Fig. 2). This rate of labelling began to fall after incubation for about 3–4 hr., and after about 6 hr. very little further increase in uptake of ^{14}C into the lipid fraction occurred (Fig. 1). Incorporation of [^{14}C]-acetate into the lipid fraction of the leucocytes is shown approx. 150 times that of the erythrocytes

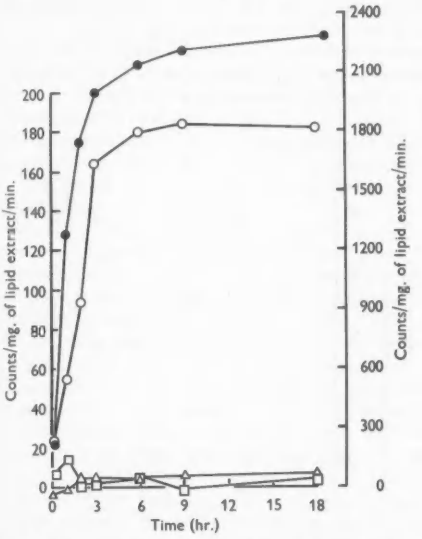

Fig. 1. Incorporation of [1-^{14}C]acetate into the lipid extract from leucocytes (●) and the plasma in which the leucocytes were incubated (○), and from erythrocytes (△) and the plasma in which the erythrocytes were incubated (□). Counts obtained from lipid extracts are plotted against time of incubation at 37° after addition of sodium [1-^{14}C]acetate. Leucocyte counts are on a scale (right-hand ordinate) 10 times that for the counts of leucocyte plasma, erythrocyte and erythrocyte plasma (left-hand ordinate). All counts are corrected to 10 000 counts/ml./min. added to the plasma before incubation.

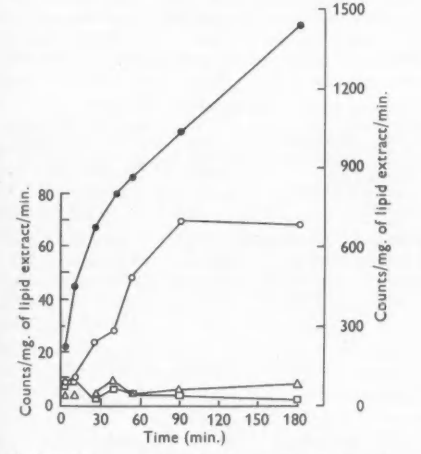

Fig. 2. Details are as given for Fig. 1. The first points are measured 2 min. after addition of the [1-^{14}C]acetate.

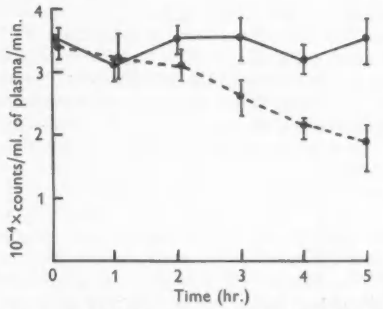

Fig. 3. Loss of [1-^{14}C]acetate from the plasma in which cells were incubated. Counts obtained from lipid extracts are plotted against time of incubation at 37° after addition of sodium [1-^{14}C]acetate. ●- - -●, Normal blood (containing leucocytes and erythrocytes; haematocrit value 42%). ●——●, Pure erythrocytes added to plasma to give a haematocrit value of 42%. Equal concentrations of [1-^{14}C]-acetate were added to each sample.

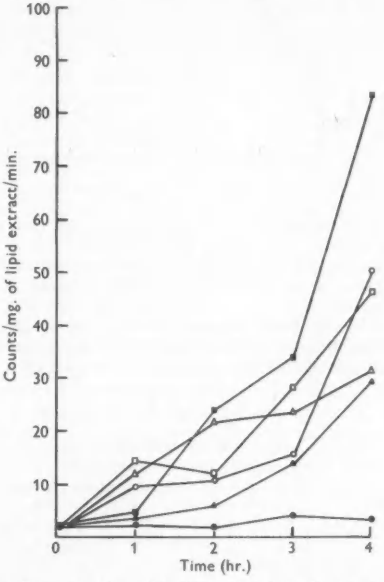

Fig. 4. Incorporation of [1-^{14}C]acetate into the total lipid extract from 1 ml. samples of: ■, normal blood incubated at 37°; ●, normal blood incubated at 4°; ○, normal blood + sodium azide to a final concentration 0·05 M; □, normal blood + dinitrophenol to a final concentration 0·1 mM; △, normal blood + potassium cyanide to a final concentration 0·1 mM; ▲, normal blood + sodium fluoride to a final concentration 0·01 M. Counts obtained from lipid extracts are plotted against time of incubation at 37° after addition of equal quantities of sodium [1-^{14}C]acetate to each sample. All counts are corrected to 10 000 counts/ml./min. added.

since counts have been expressed per mg. of lipid extracted. If the rate of incorporation of [^{14}C]-acetate is corrected for the amount of lipid extracted from erythrocytes and leucocytes (approx. 1:4), the differential rates of incorporation of [^{14}C]acetate become approx. 600:1. Similarly, the plasma counts can be corrected for the differential packed-cell volume between the erythrocytes and leucocytes when the ratio leucocyte-plasma-lipid radioactivity:erythrocyte-plasma-lipid radioactivity in Fig. 1 becomes approx. 50:1.

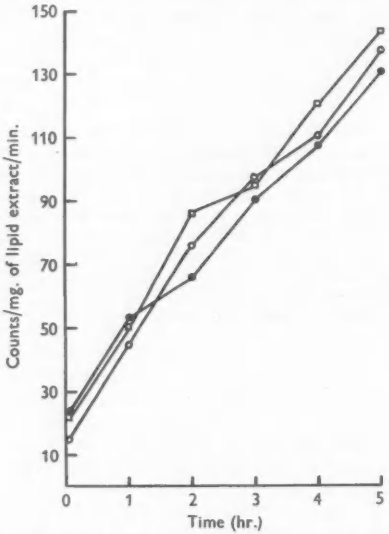

Fig. 5. Incorporation of radioactivity into the total lipid extract from 1 ml. samples of whole blood. ○, [1-^{14}C]-Acetate added to the plasma; □, [2-^{14}C]acetate added to the plasma; ●, [1-^{14}C]acetate + [2-^{14}C]acetate added to the plasma. All counts are corrected to 10 000 counts/min./ml. added to the plasma before incubation. Counts obtained from lipid extracts of whole blood are plotted against time of incubation at 37° after addition of radioactivity.

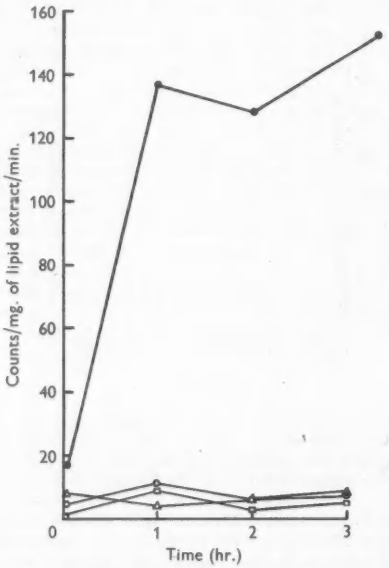

Fig. 6. Incorporation of ^{32}P into the total lipid extract from leucocytes (●) and the plasma in which the leucocytes were incubated (○), and from erythrocytes (△) and the plasma in which the erythrocytes were incubated (□). Counts obtained from lipid extracts are plotted against time of incubation at 37° after addition of [^{32}P]orthophosphate. All counts are corrected to 10 000 counts/min./ml. added to the plasma before incubation.

Table 1. *Synthesis of lipids by samples from whole blood*

Figures represent the weight and radioactivity of lipids extracted from cells and plasma from 1 ml. samples of blood. Erythrocytes have been removed or left in the samples. Samples were incubated for 3 hr. with sodium [1-^{14}C]acetate and lipid extracts prepared before and after incubation.

Sample	Wt. of lipid extract before incubation (mg.) (1 expt.)	Lipid extract before incubation (total counts/min.) (1 expt.)	Wt. of lipid extract after incubation for 3 hr. (mg.) (means of 5 expts.)	Lipid extract after incubation for 3 hr. (total counts/min.) (means of 5 expts.)
Total washed cells from 1 ml. of blood + 0·5 ml. of plasma	5·9	0·8	4·7 (S.D. 1·1)	110·8 (S.D. 20·9)
Total leucocytes from 1 ml. of blood + 0·5 ml. of plasma	3·5	0·8	3·3 (S.D. 0·8)	119·9 (S.D. 12·4)
Plasma (0·5 ml.) from total washed cells from 1 ml. of blood + 0·75 ml. of plasma	3·5	0·3	3·1 (S.D. 0·3)	19·7 (S.D. 1·0)
Plasma (0·5 ml.) from leucocytes of 1 ml. of blood + 0·75 ml. of plasma	3·6	−1·2	3·4 (S.D. 0·5)	21·2 (S.D. 3·2)

Cell-free plasma incubated with [^{14}C]acetate showed no labelling of the lipid fraction.

The total plasma ^{14}C radioactivity fell during the incubation as acetate was utilized by the cells (Fig. 3). Samples containing erythrocytes alone utilized very little, if any, of the [^{14}C]acetate during incubation for 5 hr. (Fig. 3). Calculation for whole blood showed that 2·8% (S.D. 0·65) of the radioactivity disappearing from the plasma appeared in the cell lipids.

Uptake of ^{14}C into the lipid fraction was completely inhibited by incubation at 4° and partially inhibited by glycolytic- or respiratory-enzyme inhibitors, such as sodium fluoride, sodium azide, dinitrophenol and potassium cyanide (Fig. 4).

In order to determine whether erythrocytes can synthesize lipid from acetate the incorporation of [^{14}C]acetate into the lipid fraction of the total leucocytes from 1 ml. samples of blood, obtained by haemolysing the erythrocytes with saponin (Schwartz *et al.* 1956), was compared with the rate of incorporation of the total cells (erythrocytes and leucocytes) from 1 ml. samples of the same blood.

Table 1 shows that after incubation for 3 hr. the lipid extract from both the total samples (cells + plasma) and the plasma alone contained the same radioactivity whether or not the erythrocytes were present.

If sodium [2-^{14}C]acetate was added to blood before incubation in the same quantities as was normally used for [1-^{14}C]acetate the same amount of labelling occurred in the lipid fraction (Fig. 5). Extracts of lipid from leucocytes incubated with [^{32}P]orthophosphate also showed uptake of radioactivity, presumably into their phospholipids (Fig. 6).

DISCUSSION

The rapid metabolic rate of leucocytes is well known; they have $Q_{O_2} - 32{\cdot}6$ μl. of oxygen/mg. dry wt./hr. as compared with Q_{O_2} of liver, $-11{\cdot}6$ μl. (Wagner & Sparaco, 1958), and all metabolic functions studied have confirmed this (Lawrence, 1955; Valentine, 1956; Beck, 1957, 1958; Winzler, 1958). The results reported here show that leucocytes are also very active in lipid synthesis.

It has been shown that in normal blood all the lipid synthesis is carried out by leucocytes, since the same rate of uptake of ^{14}C occurs whether the erythrocytes are present or not. Also erythrocytes appear not to be able to metabolize acetate, which is in agreement with their known lack of Krebs-cycle enzymes. The synthesis of lipid by reticulocytes (Marks & Gellhorn, 1959) is not demonstrated or disproved by the findings, since all blood used had a normal low reticulocyte count and these cells were discarded in the leucocyte and erythrocyte separation.

The lack of complete inhibition of lipid synthesis by enzyme inhibitors agrees with the findings of McKinney & Martin (1956) and McKinney, Martin, Rundles & Green (1953), who showed that sorbose 1-phosphate, oxamic acid, sodium fluoride, potassium cyanide, dinitrophenol and fluoroacetate did not completely depress leucocyte respiration.

The finding of labelled lipid in the plasma in which the leucocytes were incubated could be due either to lipids being released from living leucocytes into the plasma or to lysis of leucocyctes during incubation after they have synthesized labelled lipid. The former is the more likely explanation, since the time course of the appearance of plasma lipid follows that of the cell lipids. If the plasma-labelled lipid were due to death of leucocytes the course of plasma-labelled lipid would be expected to rise as more leucocytes lyse.

If after incubation for 19 hr. a stained smear of the leucocytes is examined, very little debris is seen; the cells all appear intact although their cytoplasm is somewhat amorphous. Unfortunately it is impossible to count the cells before and after incubation since they clump during incubation and attempts to disperse them by agitation result in lysis of large numbers of cells.

Leucocytes remain viable *in vitro* for up to 4 days (Malec & Zakrzewski, 1957). If living leucocytes can lose lipid to the plasma, this may be an important source of the plasma lipid. James *et al.* (1957) have shown that lipid synthesized by whole blood becomes attached to the plasma proteins, mainly to the α-globulins.

SUMMARY

1. Synthesis of lipids by human leucocytes *in vitro* has been demonstrated; the amount of synthesis is sufficient to account for all the lipid synthesized by whole blood.

2. It is probable that all earlier reports of lipid synthesis by erythrocytes were due to leucocyte contamination.

This work was done during the tenure of an Undergraduate Studentship of London University. I am grateful to Dr T. A. J. Prankerd for his encouragement and help, and to Professor M. L. Rosenheim for providing the facilities for this work.

REFERENCES

Altman, K. I. (1953). *Arch. Biochem. Biophys.* **42**, 478.
Altman, K. I., Watman, R. N. & Salomon, K. (1951). *Arch. Biochem. Biophys.* **33**, 169.
Beck, W. S. (1957). *Univ. Calif., Los Angeles: Sch. Med. Atomic Energy Rep.* no. 414.
Beck, W. S. (1958). *Ann. N.Y. Acad. Sci.* **75** (1), 4.
Boyd, E. M. (1936). *Arch. Path.* **21**, 739.

Burt, N. S. & Rossiter, R. J. (1950). *Biochem. J.* **46**, 569.
Davis, V. E., Spun, C. L. & Wilson, W. L. (1958). *Blood*, **13**, 367.
Fleming, A. (1926). *Brit. J. exp. Path.* **7**, 281.
Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957). *J. biol. Chem.* **226**, 497.
Granick, S. (1949). *Blood*, **4**, 404.
Jago, M. (1956). *Brit. J. Haematol.* **2**, 439.
James, A. T., Lovelock, J. E. & Webb, J. (1957). *Biochem. J.* **66**, 60P.
Lawrence, J. S. (1955). *J. Amer. med. Ass.* **157**, 1212.
Lovelock, J. E. (1955). *Biochem. J.* **60**, 692.
McKane, A. & Ingram, M. (1957). *Univ. Rochester Atomic Energy Proj.* no. 490.
McKinney, G. R. & Martin, S. P. (1956). *Blood*, **11**, 455.
McKinney, G. R., Martin, S. P., Rundles, R. W. & Green, R. (1953). *J. appl. Physiol.* **5**, 335.
Malec, J. & Zakrzewski, K. (1957). *Nature, Lond.*, **180**, 551.
Marks, P. A. & Gellhorn, A. (1959). *Fed. Proc.* **18**, 281.
Marks, P. A., Johnson, A. B., Hirschberg, E. & Banks, J. (1958). *Ann. N.Y. Acad. Sci.* **75** (1), 95.
Munn, J. I. (1958). *Brit. J. Haematol.* **4**, 344.
Peters, J. P. & van Slyke, D. D. (1946). *Quantitative Clinical Chemistry, Interpretations*, vol. 1, p. 469. London: Baillière, Tindall and Cox Ltd.
Ponder, E. (1954). *Blood*, **9**, 227.
Prankerd, T. A. J. (1958). *J. Physiol.* **143**, 325.
Rowe, C. E. (1959). *Biochem. J.* **71**, 1P.
Schwartz, S., Grazia, L., Cheney, K., Hasenfus, M. B. & Cohen, S. (1956). *U.S. Atomic Energy Comm. Biological effects of external X and Y irradiation*, T.I.D. 5220, 346.
Turner, J. C. (1958). *Arch. intern. Med.* **101**, 310.
Valentine, W. N. (1956). *Progr. Haematol.* **1**, 293.
Valentine, W. N. (1958). *Meth. med. Res.* **7**, 147.
Wagner, R. & Sparaco, R. (1958). *Ann. N.Y. Acad. Sci.* **75** (1), 16.
Wintrobe, M. M. (1956). *Clinical Haematology*, 4th ed., p. 122. London: H. Kimpton.
Winzler, R. J. (1958). *Ann. N.Y. Acad. Sci.* **75** (1), 37.

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Selective Acetylation of the Terminal Amino Group of Corticotrophin

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The amino acid sequence in α-melanocyte-stimulating hormone (α-MSH) is identical with that of the *N*-terminal tridecapeptide in corticotrophin (I) (Harris & Lerner, 1957; Harris, 1959). α-MSH (II) differs from the latter in having an *N*-acetyl substituent on its *N*-terminal serine, and an amide group on its *C*-terminal valine.

terminal serine in corticotrophin, which is essential for its adrenocorticotrophic activity, may also be one of the factors which inhibit the potential melanocyte-stimulating activity of the corticotrophin molecule. It was therefore of interest to determine whether selective *N*-acetylation of the *N*-terminal serine residue in corticotrophin would

Ser.Tyr.Ser.Met.Glu.His.Phe Arg.Try.Gly.Lys.Pro.Val.Gly...^{39}Phe
(I)

N-Acetyl Ser.Tyr.Ser.Met.Glu.His.Phe.Arg.Try.Gly.Lys.Pro.(Val.NH_2)
(II)

This common sequence, part of which is also found in β-melanocyte-stimulating hormone (β-MSH), provides an explanation in terms of chemical structure for the intrinsic melanocyte-stimulating activity of corticotrophin reported by Bell (1954) and Dixon (1956*a*). However, although it contains the entire amino acid sequence of α-MSH within its structure, corticotrophin possesses melanocyte-stimulating activity of only 1% (or less) of that of α-MSH, on a weight basis.

Harris (1958, 1959) suggested that the free *N*-

diminish its corticotrophic activity and also enhance its melanocyte-stimulating activity.

The *N*-terminal serine of corticotrophin cannot be selectively *N*-acetylated by the usual methods of acylation, since the ε-amino groups of the four lysine residues in the molecule would also be acetylated under the same conditions.

In their account of the synthesis of α-MSH peptide fragments, Guttmann & Boissonnas (1958) reported that treatment of the protected pentapeptide benzyloxycarbonylSer.Tyr.Ser.Met.α-methyl-γ-benzylGlu with a solution of hydrogen bromide in acetic acid, to remove the benzyloxy-

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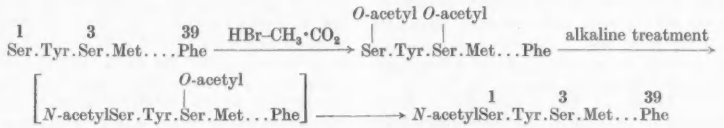
carbonyl group, resulted in *O*-acetylation of both serine residues. On adjustment of the pH above 7, *N*-acetylSer.Tyr.*O*-acetylSer.Met.α-methyl-γ-benzylGlu was obtained, as a result of an O → N acyl shift. The preparation of *O*-acetylserine by a similar reaction, with hydrochloric acid in acetic acid, was reported by Sheehan, Goodman & Hess (1956).

O-Acyl substituents of hydroxyamino acids, which are stable under acid conditions, are easily hydrolysed on exposure to alkali, provided that the amino function is not free to participate in the acyl-migration reaction (Synge, 1939).

These observations provide the basis for a method of selective *N*-acetylation of the *N*-terminal serine in corticotrophin, through the following sequence of reactions:



Corticotrophin A_1 (Dixon & Stack-Dunne, 1955) was used as a starting material. Conditions of alkali exposure were chosen which produced the O → N acyl shift on the serine in position 1, the hydrolysis of the *O*-acetyl group on the serine in position 3, and the removal of amide ammonia to convert corticotrophin A_1 into A_2 in a single step.

The resulting modified corticotrophin, referred to below as *N*-acetylcorticotrophin A_2, has been purified by ion-exchange chromatography, characterized and assayed for melanocyte-stimulating and corticotrophic activity.

EXPERIMENTAL

Materials

Corticotrophin A_1. This was prepared by the method of Dixon & Stack-Dunne (1955) with minor modifications. Frozen pig-pituitary glands were used in place of acetone-dried glands so as to prevent the conversion of corticotrophin A_1 into A_2. Ion-exchange chromatography was performed with 0·25% of chlorbutol (1:1:1-trichloro-2-methylpropan-2-ol) in the place of phenol as a bacteriostat in the buffer so that polypeptide could be detected by its absorption of ultraviolet radiation. The product was obtained from the phosphate buffer solution by the desalting method of Dixon (1959) described below.

Corticotrophin A_2. This was prepared by alkaline treatment of corticotrophin A_1 (Dixon & Stack-Dunne, 1955; Li, Geschwind, Dixon, Levy & Harris, 1955; Shepherd, Howard *et al.* 1956) with the conditions described below for the treatment of *O*-acetylated corticotrophin A_1. The product was isolated after ion-exchange chromatography.

α-Melanocyte-stimulating hormone. This was required to compare the *N*-terminal fragments released by trypsin digestion of α-MSH and *N*-acetylcorticotrophin A_2. It was prepared by one of us (J.-P.W.), using a melanocyte-stimulating hormone (MSH)-concentrate (Armour, lot D 14083–4) as starting material. The first step in the purification was essentially the method described by Steelman, Andersen & McGregor (1959).

MSH-concentrate (2·5 g.) was twice extracted with 0·01 M-acetic acid (25 ml.). The insoluble material (20–23% by weight) was removed by centrifuging, and the clear supernatant adjusted to pH 6·5 with aq. NH_3. After removal of the precipitate, the supernatant (containing 65–70% by weight of the starting material) was placed on a column of carboxymethylcellulose (0·5 m-equiv./g.) equilibrated with 0·01 M-ammonium acetate, pH 6·5. β-MSH was not retained under these conditions. After washing with 0·01 M-buffer, the α-MSH was eluted with 0·1 M-ammonium acetate buffer, pH 6·5. The peak material was desalted on Amberlite IRC-50 (Dixon, 1959), freeze-dried and reapplied on carboxymethylcellulose equilibrated with 0·01 M-ammonium acetate, pH 6·5. It was eluted with a gradient of 0·1 M-ammonium acetate (pH 6·5), by using a mixing chamber of 250 ml.

Although Steelman *et al.* (1959) reported that a similar procedure gave highly pure α-MSH, our product was heterogeneous and was purified further by preparative paper chromatography, with the solvent system butan-1-ol–acetic acid–water–pyridine (30:6:24:20, by vol.) described by Waley & Watson (1953) (butan-1-ol was purified by the method of Clarke, Robinson & Smith, 1927). It was applied on Whatman no. 3 paper (pre-washed with 33% acetic acid) at a concentration of 1 mg./cm., for an ascending run of 10 hr. α-MSH, which migrated as the fastest-moving component (R_F 0·69), was recovered by elution with 5% acetic acid, followed by freeze-drying of the eluate.

β-Melanocyte-stimulating hormone. This was prepared from pig pituitary glands by ion-exchange chromatography (Dixon, 1960).

Hydrogen bromide in acetic acid. For the requirements of the reaction with corticotrophin, it was essential to prepare this reagent free from all traces of bromine.

Bromine was added dropwise into a filter-flask containing anhydrous tetralin, through a separating funnel whose stem reached below the surface of the liquid. The HBr gas generated was passed successively through a wash-bottle containing tetralin, a tube filled with copper turnings and a second wash-bottle containing tetralin, to remove the last traces of bromine. The gas was finally absorbed into acetic acid in a wash-bottle covered with black paper, externally cooled to compensate for warming during absorption, and protected from moisture by means of a drying tube containing $CaCl_2$. Polyethylene tubing was used for all connexions.

The rate of flow of HBr was regulated by gentle heating of the flask containing tetralin, and absorption continued until a concentration of approximately 3·5 N-HBr was reached. Samples of this colourless and slightly viscous solution were removed to determine the concentration of

HBr by titration, and the remainder diluted with acetic acid and redistilled thioglycollic acid to a final concentration of 2·8 N-HBr and 5% (by vol.) of thioglycollic acid. This reagent was immediately used for reaction with corticotrophin.

On standing, the reagent developed a yellow colour within a few hours, which increased progressively. A new batch was prepared for each acetylation reaction.

Ammonium bicarbonate buffer (pH 10·0). This was prepared by mixing 0·2 N-NH_4HCO_3 (100 ml.) with aq. 0·8 N-NH_3 solution (100 ml.).

Methods

Treatment of corticotrophin A_1 with hydrogen bromide in acetic acid. A freshly prepared solution (1·5 ml.) of 2·8 N-HBr in acetic acid containing 5% of thioglycollic acid was added to corticotrophin A_1 (12·0 mg.; approx. 2·5 μM) in a small, glass-stoppered test tube covered with dark paper. Complete dissolution was achieved by shaking gently by hand for about 30 min. After a total reaction time of 75 min. at 25°, the tube was rapidly connected to a water pump to remove most of the HBr, and the material immediately precipitated by addition of anhydrous, peroxide-free ether (10 ml.). After centrifuging and removal of the supernatant, the white precipitate was resuspended in ether (10 ml.), the suspension centrifuged, and the supernatant discarded. The ether wash was repeated twice more to ensure complete removal of thioglycollic acid. The precipitate was freed from last traces of ether *in vacuo*, dissolved in water (1·5 ml.) and freeze-dried. The recovery of *O*-acetylated corticotrophin A_1 was quantitative (14·0 mg.; approx. 2·5 μM, accounting for the addition of 9 moles of HBr and 2 acetyl groups).

Alkali-exposure of O-acetylated corticotrophin A_1. (*a*) Aq. NH_3 soln. (1·0 M; pH 11·5; 1 ml.) was added to *O*-acetylated corticotrophin A_1 (10·0 mg.) in a small glass-stoppered test tube, and the resulting clear solution held at room temperature for 6 hr. The material was then recovered quantitatively by freeze-drying, and used directly for purification by ion-exchange chromatography on Amberlite IRC-50.

(*b*) Ammonium bicarbonate buffer at pH 10 (1·5 ml.) was added to *O*-acetylated corticotrophin A_1 (14·0 mg.), and the clear solution held at room temperature for 12 hr. The material was recovered and treated further as described in (*a*).

Ion-exchange chromatography on Amberlite IRC-50. The details were those described by Hirs, Moore & Stein (1953) and Dixon & Stack-Dunne (1955). The stock solution of buffer used for the isolation of corticotrophin A_1 had a composition of 0·1 M-Na_2HPO_4, 0·1 M-NaH_2PO_4 and 0·25% (w/v) of chlorbutol. The buffer used for chromatography of the product of acetylation was a dilution of the stock buffer to three-quarters of its strength ([Na^+], 0·225 N). All columns were equilibrated before use so that the effluent possessed the same pH value as the influent within 0·01 unit.

Isolation of products after chromatography (desalting). The method of Dixon (1959) was used. The solution was acidified by the addition of 5% (v/v) acetic acid and run through a small column of the acid form of finely ground carboxylic resin Amberlite IRC-50, which adsorbed the product. The resin was washed with 5% acetic acid until the effluent was salt-free, and the product displaced with 50% acetic acid. It was obtained in the dry state by diluting the 50% acetic acid effluent with water and drying while frozen. The other operations were carried out in the cold (4°).

N- and C-Terminal amino acid analyses. *N*- and *C*-Terminal group analyses were carried out on samples of corticotrophin A_1, corticotrophin A_2 and *N*-acetylcorticotrophin A_2 in a series of parallel experiments.

The *N*-terminal group was determined on samples of 2·5 mg. (approx. 0·5 μM) by the phenyl *iso*thiocyanate method (Edman, 1950) according to the paper-strip procedure described by Harris & Li (1954) for sheep corticotrophin and by Harris & Roos (1959) for β-MSH.

The *C*-terminal analyses were performed by digesting samples of 0·6 mg. (approx. 0·12 μM) with carboxypeptidase, according to the method described by Fraenkel-Conrat, Harris & Levy (1955).

Trypsin digestion of corticotrophin A_2, N-acetylcorticotrophin A_2 and α-melanocyte-stimulating hormone. Corticotrophin A_2 (2·5 mg.; approx. 0·5 μM) in 5 mM-2-amino-2-hydroxymethylpropane-1:3-diol (tris)–HCl buffer at pH 7·8 (2·5 ml.) was treated with 10 μl. of enzyme solution [Boehringer trypsin: 0·3% soln. in 0·02 N-HCl which had been heated to 37° for 24 hr. at 1% concentration in 0·06 N-HCl to reduce chymotryptic contamination (cf. Northrop & Kunitz, 1936; Redfield & Anfinsen, 1956); enzyme–substrate ratio, 1:85]. The stoppered solution was kept at 25° for 3 hr., then acidified to pH 4 and freeze-dried.

N-Acetylcorticotrophin A_2 was digested in a similar manner. It was relatively insoluble at pH 7·8, but a clear solution was obtained within 1 hr. after the addition of enzyme. The solution was kept at 25° for 4 hr.

α-MSH (0·8 mg.; approx. 0·5 μM) in 5 mM-tris–HCl buffer at pH 7·8 (1 ml.) was treated with 3 μl. of the enzyme solution (enzyme–substrate ratio, 1:90). After 3 hr. at 25°, the solution was acidified to pH 4 and freeze-dried.

Separation of the peptide fragments derived from trypsin digestion. Each digest (from approx. 0·5 μM of substrate) was applied on Whatman no. 1 paper as a narrow, 8 cm. long line, and was submitted to high-voltage ionophoresis (Michl, 1951) in pyridine acetate buffer at pH 6·5 (pyridine–acetic acid–water, 10:0·4:90, by vol.) for 75 min. at 2000 v.

The peptide bands were located by staining narrow guide strips with ninhydrin and with tyrosine-, histidine- and arginine-specific reagents (see Block, Durrum & Zweig, 1955). From each paper, the only band containing both tyrosine and histidine and corresponding to the *N*-terminal octapeptide sequence Ser.Tyr.Ser.Met.Glu.His.Phe.Arg was eluted with 5% acetic acid. The three solutions were evaporated to dryness, and the recovered materials were used for further characterization of the *N*-terminal octapeptide from each digest.

Biological assays. All biological assays were performed on twice-chromatographed *N*-acetylcorticotrophin A_2. Corticotrophic activity was measured by adrenal ascorbic acid depletion of hypophysectomized rats by the method of Sayers, Sayers & Woodbury (1948).

MSH-activity was assayed in the illuminated intact frogs, according to the method of Landgrebe & Waring (1950). Assays on the isolated frog skin were performed by Dr Margaret R. Wright and Dr Aaron B. Lerner, using the method of Shizume, Lerner & Fitzpatrick (1954).

RESULTS

Chromatographic patterns

The products of the sequence of reactions described separated on chromatography into components as shown in Figs. 1 and 3. Of the ultraviolet-absorbing material applied on the column, 34–45% was recovered in the main peak, whose characterization as *N*-acetylcorticotrophin A_2 is described below.

On rechromatography of the main peak (*N*-acetylcorticotrophin A_2), 87% of the absorption applied (91% of the absorption recovered from the column) emerged in the main peak (Fig. 2).

Experiments were performed in which a lower pH was used for the last stage of reaction. When *O*-acetylated corticotrophin A_1 (14·0 mg.) treated with ammonium bicarbonate buffer at pH 10 (1·5 ml.) for 12 hr. was chromatographed, a fairly similar pattern was obtained (Fig. 3).

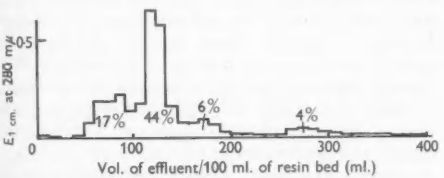


Fig. 1. Chromatography of the reaction products obtained by alkaline treatment (0·1 N-NH_3 soln., pH 11·5, for 6 hr.) of *O*-acetylated corticotrophin A_1. A column (41·5 cm. × 0·6 cm.²) of carboxylic resin Amberlite IRC-50 (XE-64) was equilibrated and developed with a buffer containing 0·075 M-NaH_2PO_4 and 0·075 M-Na_2HPO_4; load, 7·3 mg. The figures under the peaks express the yields with respect to the total ultraviolet absorption applied to the column. The total recovery was 70%.

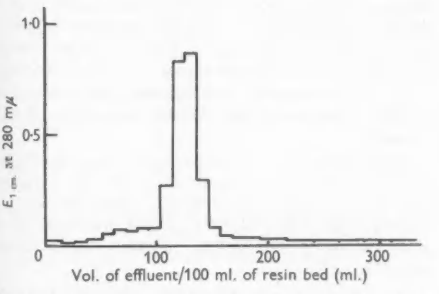


Fig. 2. Rechromatography of *N*-acetylcorticotrophin A_2. A column (41·3 cm. × 0·6 cm.²) of carboxylic resin was equilibrated and developed with sodium phosphate buffer as in Fig. 1; load, 4·6 mg. Of the ultraviolet absorption applied, 87% (91% of the absorption recovered) emerged in the main peak.

Alkaline treatment at a still lower pH [pH 9·0, 0·1 M-sodium bicarbonate buffer for 60 hr., used by Shepherd, Howard *et al.* (1956) for removing amide ammonia from β-corticotrophin] resulted in a higher yield of the peak having a retention volume of 150–160 ml./100 ml. of resin bed.

Further exposure to alkali (0·1 M-ammonium bicarbonate buffer at pH 10 for 10 hr.) of the peak at 150–160 ml./100 ml. resin bed produced *N*-acetylcorticotrophin A_2, as shown by rechromatography. Both its position on the chromatogram and its conversion into *N*-acetylcorticotrophin A_2 on further treatment with alkali suggest that this material is *NO*-diacetylcorticotrophin A_2. This further conversion increases the possible yield of *N*-acetylcorticotrophin A_2 to 50% of the u.v.-absorbing material applied (65% of that emerging). The rest of the absorption recovered is accounted for partly by the unacetylated corticotrophin A_2 and mainly by the peak at 60–70 ml./100 ml. of resin bed.

In attempts to identify the material from the peak at 60–70 ml./100 ml. of resin bed, separate samples were rechromatographed after the following treatments: (i) further exposure to alkali (ammonium bicarbonate buffer, 0·1 M, pH 10 for 12 hr.); (ii) reduction with thioglycollic acid (0·2 M, pH 3·5, 70° for 8 hr.) (Dedman, Farmer & Morris, 1955, 1957; Dixon, 1955, 1956*a*). Both treatments failed to produce *N*-acetylcorticotrophin A_2 as shown by rechromatography. No further attempt was made to characterize this material.

Corticotrophin A_1 emerged, in the system used, as a peak with retention volume of about 500 ml./100 ml. of resin bed. After treatment of corticotrophin A_1 with ammonia solution (M for 6 hr.), the result shown in Fig. 4 was obtained. The main peak at about 280 ml./100 ml. of resin bed is corticotrophin A_2. Unfortunately, minor components emerged with the same retention volume as

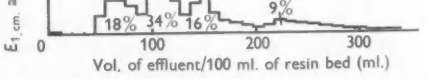


Fig. 3. Chromatography of the reaction products obtained by alkaline treatment (ammonium bicarbonate buffer, pH 10, for 12 hr.) of *O*-acetylated corticotrophin A_1. A column (45·5 cm. × 0·6 cm.²) of carboxylic resin equilibrated and developed with sodium phosphate buffer as in Fig. 1; load, 7·7 mg. The figures under the peaks express the yields with respect to the total ultraviolet absorption applied to the column. The total recovery was 78%.

N-acetylcorticotrophin A_2. The nature of these minor components has not been established. (Some of the corticotrophin A_1 used for these preparations has since been found to contain chromatographically less-retarded components which were produced during desalting when a particular batch of acetic acid was used. The impurities in the corticotrophin A_2 may be related to such components.) Their existence means that the *N*-acetylcorticotrophin A_2 peak may be slightly contaminated with biologically-active unacetylated derivatives of corticotrophin. If, however, these minor components still possess an *N*-terminal serine residue, after the acetylation reaction they should be largely replaced by the corresponding *N*-acetyl derivatives, which should be less retarded in the chromatographic system than *N*-acetylcorticotrophin A_2, so that the degree of contamination should be small. These *N*-acetyl derivatives may make up part of the peak observed at 60–70 ml./100 ml. of resin bed. There is chromatographic evidence that the material of this peak is heterogeneous.

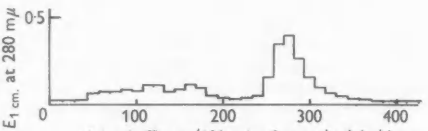


Fig. 4. Chromatography of the reaction products obtained by alkaline treatment (M-NH_2 soln. for 6 hr.) of corticotrophin A_1. A column (44·5 cm. × 0·6 cm.²) of carboxylic resin was equilibrated and developed with sodium phosphate buffer as in Fig. 1; load, 6·6 mg. Of the ultraviolet absorption applied, 55% emerged in the corticotrophin A_2 peak. The total recovery was 87%.

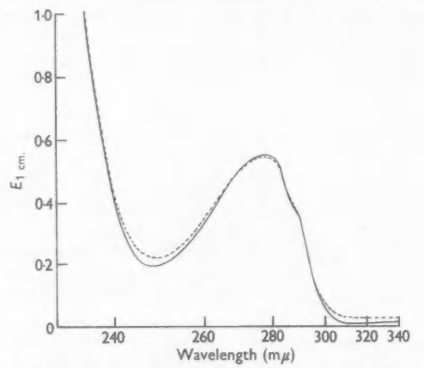


Fig. 5. Ultraviolet-absorption spectra of corticotrophin A_1 and *N*-acetylcorticotrophin A_2. ——, Corticotrophin A_1; - - - - -, *N*-acetylcorticotrophin A_2. Concentration: 1·05 mg. in 3·0 ml. of 2% (v/v) acetic acid solution. $E_{1\,cm.}$ at 278 mμ, 0·550.

Characterization of N-acetylcorticotrophin A_2

Ultraviolet-absorption spectrum. The ultraviolet-absorption spectra of desalted corticotrophin A_1 and *N*-acetylcorticotrophin A_2 were compared for solutions of 1·05 mg. in 3·0 ml. of 2% acetic acid (Fig. 5), with a Beckman Automatic Recorder. The extinction reading at the absorption maximum (278 mμ) was identical for the two compounds, corresponding to a value of $E^{1\%}_{1\,cm.}$ of 16·0.

N- and C-Terminal group analyses. No free *N*-terminal group was detected when the phenyl*iso*-thiocyanate method was applied to *N*-acetylcorticotrophin A_2. Control experiments with corticotrophins A_1 and A_2 yielded an average of 0·65 mole of serine as *N*-terminal amino acid.

There was no indication of modification of the *C*-terminal end of the molecule when the amino acids released by carboxypeptidase digestion of *N*-acetylcorticotrophin A_2 and corticotrophins A_1 and A_2 were compared. Paper chromatography in the solvent system butanol–acetic acid–water (4:1:5, by vol.), followed by development with ninhydrin, revealed the presence of phenylalanine and two weaker spots of glutamic acid and leucine, with comparable intensities in all three samples.

Trypsin digestion. When corticotrophin is digested with the enzyme trypsin, the *N*-terminal octapeptide Ser. Tyr. Ser. Met. Glu. His. Phe. Arg is released (Bell, 1954). Under similar conditions, the corresponding *N*-acetyl fragment is released from α-MSH (Harris, 1959).

Trypsin digestion was used to compare the *N*-terminal octapeptide released from *N*-acetylcorticotrophin A_2 with those from corticotrophin A_2 and α-MSH. Fig. 6 shows the pattern obtained when digests of the three substrates were submitted to paper ionophoresis in pyridine acetate buffer at pH 6·5, in a similar run. Ionophoresis at this pH provided the best means of resolving the *N*-terminal octapeptide from the other basic and acidic fragments released during trypsin digestion of each substrate.

Peptide A (from corticotrophin A_2) was slightly basic at pH 6·5, compatible with the structure Ser. Tyr. Ser. Met. Glu. His. Phe. Arg. It gave a brown spot with ninhydrin and in addition responded positively to the tyrosine, histidine and arginine colour-tests.

No bands of corresponding mobility were observed with the digests of *N*-acetylcorticotrophin A_2 and α-MSH. Instead, a band of a slightly acidic substance, compatible with the structure *N*-acetylSer. Tyr. Ser. Met. Glu. His. Phe. Arg, appeared with both digests (peptides B and C re-

spectively). These bands gave no colour with ninhydrin, but responded positively to the tyrosine, histidine and arginine colour-tests.

Apart from the difference in bands A and B, the trypsin digests of corticotrophin A_2 and *N*-acetylcorticotrophin A_2 were resolved into an identical pattern on ionophoresis at pH 6·5.

Further characterization of bands A, B and C was achieved by comparing the R_F of the three peptides by chromatography on paper, in the solvent system butanol–acetic acid–water (4:1:5, by vol.). On development with the tyrosine reagent, peptide B (from *N*-acetylcorticotrophin A_2) gave an intense spot with an R_F of 0·43 and a minor spot with an R_F of 0·26 which was identified as the corresponding methionine sulphoxide peptide. Peptides containing methionine normally separated into two components when chromatographed in the solvent system butanol–acetic-acid–water (4:1:5, by vol.), as a result of partial oxidation of the methionine into methionine sulphoxide during operations (see Harris & Roos, 1959). Peptide C (from α-MSH) gave an identical pattern, whereas peptide A (from corticotrophin A_2) revealed the same pair of spots at much lower R_F values (0·17 and 0·09 respectively), as expected from the absence of the *N*-terminal acetyl group.

Finally, peptides A, B and C were analysed for amino acid composition. Samples were hydrolysed with 5·7 N-HCl for 16 hr., and the amino acids identified by paper chromatography in the solvent system butanol–acetic acid–water (4:1:5, by vol.), and by paper ionophoresis in 2·5% formic acid at pH 2·1. All three samples were found to contain the expected amino acids (Ser, Tyr, Ser, Met, MetSO, Glu, His, Phe and Arg).

Chromatography on paper. Samples of desalted *N*-acetylcorticotrophin A_2 and corticotrophin A_2 (0·25 mg. of each) were applied on Whatman no. 1 paper for chromatography in the solvent system butanol–acetic acid–water–pyridine (30:6:24:20, by vol.) described by Waley & Watson (1953). Corticotrophin A_2 migrated as a slightly elongated spot with an R_F of 0·35, while *N*-acetylcorticotrophin A_2 moved as a single, fairly compact spot with an R_F of 0·50. This high value agrees with the presence of an *N*-terminal acetyl group.

Biological assays

Corticotrophin assays. Table 1 shows that *N*-acetylcorticotrophin A_2 possessed less than 10% of the activity of corticotrophin A_1. The slight activity found (about 2%) could be intrinsic, but could also be due to slight contamination with non-acetylated products, such as those with the same retention volume produced by the action of alkali on corticotrophin A_1 (Fig. 4).

Melanocyte-stimulation assay. (*a*) In the intact frog *N*-acetylcorticotrophin A_2 did not show increased activity compared with corticotrophin A_1 if the melanocyte index was read up to 1 hr. after injection, and was probably less active at 0·5 hr. (Table 2). But its action was prolonged, so that if the comparison was made later (6 or 18 hr.) it had

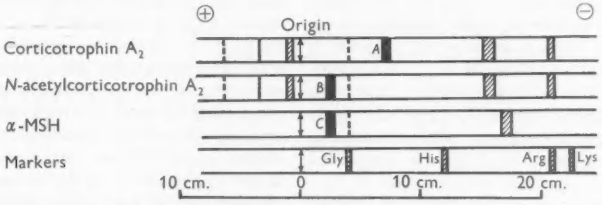


Fig. 6. Ionophoresis diagrams of products of tryptic hydrolysis of corticotrophin A_2, *N*-acetylcorticotrophin A_2 and α-MSH. Ionophoresis was carried out in pyridine acetate buffer (pH 6·5) at 35–40 v/cm. for 75 min.

Table 1. *ACTH assay of* N-*acetylcorticotrophin* A_2*: depletion of ascorbic acid in hypophysectomized rats*

The estimate of the standard deviation of the responses was 16 (9 degrees of freedom) and it was 15 if another 6 rats (another 4 degrees of freedom) which were used in the same assay for another test were also included. The intravenous injection was 0·25 ml./100 g. rat weight. Corticotrophins A_1 and A_2 do not differ significantly in ACTH activity (Dixon & Stack-Dunne, 1955; Shepherd, Howard *et al.* 1956).

Substance	Dose (μmg./ml.)	No. of rats	Mean change of adrenal concn. of ascorbic acid (mg./100 g. of tissue)
Corticotrophin A_1	40	4	−49
N-Acetylcorticotrophin A_2	4000	3	−82
	400	3	−1
	40	3	+16

Table 2. *Melanocyte-stimulating assay of N-acetylcorticotrophin A_2 on intact frogs*

Substance	Dose (μmg.)	No. of frogs	Mean melanocyte index: Before injection	½ hr.	1 hr.	6 hr.	18 hr. (After injection)	Mean difference from initial melanocyte index: ½ hr.	1 hr.	6 hr.	18 hr. (After injection)
N-Acetylcorticotrophin A_2	500	4	1·7	3·0	3·9	4·6	3·4	1·3	1·9	2·9	1·7
	150	3	1·7	2·8	3·6	2·4	—	1·1	1·7	0·7	—
Corticotrophin A_1*	5000	4	1·4	3·4	3·9	3·5	1·9†	2·0	2·5	2·1	0·5
	1500	3	1·5	3·3	3·9	3·3	—	1·8	2·4	1·8	—
	500	3	1·8	3·5	3·7	2·6	—	1·7	1·9	0·8	—
β-Melanocyte-stimulating hormone	15	4	1·4	3·8	3·9	1·4	—	2·3	2·4	−0·1	—
	5	3	1·6	3·3	3·8	1·3	—	1·7	2·2	−0·3	—
Standard deviation ...			0·24	0·35	0·47	0·63	0·50				

* Corticotrophins A_1 and A_2 are reported to have the same MSH activity (Shepherd, Howard *et al.* 1956).
† One frog (index 1·6) dead; mean of remainder, 2·0.

more than 10 times the activity of corticotrophin A_1. In contrast, β-MSH showed much higher activity than corticotrophin A_1 mainly at short time intervals after injection, about 100 times the activity at 1 hr., but much less than 33 times at 6 hr. The data (particularly those at 6 hr.) suggest that the potency ratio obtained depended on the dose used. This type of 'invalid' assay may be expected when substances of different properties are compared.

(*b*) Two samples of *N*-acetylcorticotrophin A_2 assayed in isolated frog skin had activity of 410 and 440 units/μg. respectively. The corresponding values for corticotrophin A_1 were 50 and 75. The activities ascribed to α-MSH and β-MSH by the same assay method are about 25 000 and 10 000 units/μg. respectively.

N-Acetylcorticotrophin A_2 thus appears to be 5–10 times as active as corticotrophin A_1 when assayed on isolated frog skin. While the melanocyte-stimulating activity of the latter is less than 0·5 % that of α-MSH, that of *N*-acetylcorticotrophin A_2 is increased to approx. 2 %, when compared on a weight basis (or to 5 % when compared on a molar basis).

DISCUSSION

The results reported suggest that selective acetylation of the α-amino group of the *N*-terminal serine residue of corticotrophin has been achieved under the conditions described.

The large fall in corticotrophin (ACTH) activity found on acetylation, together with the similar falls after other reactions of the same serine residue (White, 1955; Shepherd, Willson *et al.* 1956; Dixon, 1956*b*; Geschwind & Li, 1957), supports the view that the unsubstituted *N*-terminal serine residue is an essential constituent of a highly active ACTH molecule. As we do not know whether the slight ACTH activity found in the *N*-acetylcorticotrophin A_2 is intrinsic or due to contamination, we cannot tell if any activity is possessed when the group is blocked. If possessed, such activity could be due either to a low activity at the site of action or to a low degree of deacetylation in the animal body.

In the introduction we posed the question whether *N*-acetylcorticotrophin A_2 has greater MSH activity than has corticotrophin A_1. While there is no simple answer to this question, we can say that no spectacular increase of MSH activity has been observed. The results of the assay with isolated frog skin indicate that *N*-acetylcorticotrophin A_2 possesses about 2 % of the activity of α-MSH on a weight basis (or about 5 % on a molar basis) compared with about 0·3 % on a weight basis for corticotrophin A_1, while in the assay with intact frogs a potentiation of the same order of magnitude is apparent, but only at the longer times after injection. The high activity of β-MSH in comparison with corticotrophin is, in contrast, most marked shortly after injection and much less at longer intervals. Since some of the methods for assessing MSH action demonstrate increased activity, the idea that the free amino group inhibits this activity is supported. Anfinsen (1959) quotes Boissonnas as finding that synthetic α-MSH has about seventy times the activity of the corresponding peptide without the acetyl group.

The low order of MSH activity in *N*-acetylcorticotrophin A_2, which possesses the complete sequence of α-MSH, suggests that the structural features of the part of the corticotrophin molecule that is outside this sequence (e.g. chain length, configuration and charge distribution) seem to inhibit the potential MSH activity of the *N*-acetylcorticotrophin A_2. The diminution of activity which accompanies the replacement of the glutamyl residue of pig β-MSH by the seryl residue of ox β-MSH (Li, 1957) may be a similar inhibition of potential activity, since the locus concerned is

outside the sequence common to MSH-active substances to which activity is presumably due.

Assay methods are often invalid in the sense that the potency ratio which they yield depends on the doses of material tested. Furthermore the potency ratio may depend on the conditions of the test. For MSH activity different potency ratios may be shown under different conditions (e.g. Lock, 1952). In particular, many preparations show a prolongation of action when treated with alkali (cf. Landgrebe & Mitchell, 1954, and review by Li, 1957), so that such materials would show a higher potency against a standard if the assessment were made long after injection. From our data it appears that even the potency ratio between natural corticotrophin and β-MSH depends on the time after injection at which the effects are assessed and may also depend on the doses used. This emphasizes the necessity for specifying the conditions of assay when quoting any figure for a potency ratio.

The use of the isolated frog skin for the determination of MSH activity does not avoid this indeterminacy of potency ratio, since the treatment of some materials with alkali leads to a prolongation of action (Dr A. B. Lerner, personal communication). All the differences cannot, therefore, be due to differences in rates of absorption and excretion of the injected substances by the whole frog.

Indeterminacy of potency ratio is to be expected where substances of greatly different chemical properties exhibit a biological effect, because a series of reactions of different specificities may occur in the production of the effect. This is apart from the other invalidities in the assay of impure substances produced by interference by the impurities. The indeterminacy in testing pure substances may occur with corticotrophic potency, though the evidence is less clear than that for MSH activity. Only substances of similar molecular size possess ACTH activity, and this may explain why a unique value for the potency ratio is often obtained. Certainly the addition of other substances or peptic digestion of its molecules can prevent corticotrophin from showing its action if it is injected subcutaneously, in contrast with the full action shown by the same preparations injected intravenously. Hence assays with subcutaneous injection, e.g. that of United States Pharmacopeia XV, which are designed not to determine the amount of hormone present but to predict the effect that a preparation will have when administered intramuscularly or subcutaneously (Thompson & Fisher, 1953), give potency ratios very different from the original ascorbic acid-depletion test with intravenous injection (Sayers *et al.* 1948). The assay with subcutaneous injection allows other substances (e.g. proteolytic enzymes) to interact with the corticotrophin after injection to about the same extent that they may be expected to interact after intramuscular injection into man, and so gives a better measure of the effect likely to be obtained clinically than the test with intravenous injection, designed to measure the amount of corticotrophin present. The result of this test can be affected by adding unrelated protein (Brown, Moyer, Davies & Cox, 1955). The observation by Fortier (1958) that crude pituitary extracts from different species show different rates of absorption by adrenal tissue, as judged by its subsequent production of steroids, may mean that even pure substances would show different corticotrophic potency ratios according to the conditions of testing.

The indeterminacy of potency ratio emphasizes the difficulties of ascribing complex biological activities to particular features of molecular structure. It should be noted particularly for MSH activity, both because the variations in potency ratio can be large and because opportunities exist for studying a series of compounds of natural, chemically-modified and synthetic origins which possess MSH activity.

SUMMARY

1. The serine residues of corticotrophin A_1 were *O*-acetylated by treatment with acetic acid containing hydrogen bromide. Subsequent treatment with mild alkali moved one acetyl group to the terminal amino group, hydrolysed the other and liberated amide ammonia. The product (*N*-acetylcorticotrophin A_2) has been purified by ion-exchange chromatography and characterized.

2. *N*-Acetylcorticotrophin A_2 possesses less than 10% of the corticotrophic activity of corticotrophin A_1. This result supports suggestions that a free amino group on the *N*-terminal serine residue is essential for high corticotrophic activity.

3. *N*-Acetylcorticotrophin A_2 possesses 5–10 times the melanocyte-stimulating activity of corticotrophin A_1 when assayed on isolated frog skin.

4. In the intact frog *N*-acetylcorticotrophin A_2 possesses no more melanocyte-stimulating activity than corticotrophin A_1 if the response is assessed up to 1 hr. after injection, but more than ten times the activity if the response is assayed after 6 hr. β-Melanocyte-stimulating hormone possesses about 100 times the melanocyte-stimulating activity of corticotrophin A_1 if the response is assessed 1 hr. after injection into the frog, but much less if the assessment is made later.

5. Comparison of the results of melanocyte-stimulating assays with chemical structure suggests that the potential activity of the sequence

of α-melanocyte-stimulating hormone in *N*-acetylcorticotrophin A_2 is inhibited by other structural features of its molecule.

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REFERENCES

Anfinsen, C. B. (1959). *The Molecular Basis of Evolution*, p. 154. New York: J. Wiley and Sons Inc.
Bell, P. H. (1954). *J. Amer. chem. Soc.* **76**, 5565.
Block, R. J., Durrum, E. L. & Zweig, G. (1955). *A Manual of Paper Chromatography and Paper Electrophoresis*, pp. 87–100. New York: Academic Press Inc.
Brown, R. A., Moyer, A. W., Davies, M. C. & Cox, H. R. (1955). *Arch. Biochem. Biophys.* **58**, 68.
Clarke, J., Robinson, R. & Smith, J. C. (1927). *J. chem. Soc.* p. 2647.
Dedman, M. L., Farmer, T. H. & Morris, C. J. O. R. (1955). *Biochem. J.* **59**, xii.
Dedman, M. L., Farmer, T. H. & Morris, C. J. O. R. (1957). *Biochem. J.* **66**, 166.
Dixon, H. B. F. (1955). *Biochim. biophys. Acta*, **18**, 599.
Dixon, H. B. F. (1956*a*). *Biochim. biophys. Acta*, **19**, 392.
Dixon, H. B. F. (1956*b*). *Biochem. J.* **62**, 25P.
Dixon, H. B. F. (1959). *Biochim. biophys. Acta*, **34**, 251.
Dixon, H. B. F. (1960). *Biochim. biophys. Acta*, **37**, 38.
Dixon, H. B. F. & Stack-Dunne, M. P. (1955). *Biochem. J.* **61**, 483.
Edman, P. (1950). *Acta chem. scand.* **4**, 283.
Fortier, C. (1958). *Proc. Soc. exp. Biol., N.Y.*, **99**, 628.
Fraenkel-Conrat, H., Harris, J. I. & Levy, A. L. (1955). In *Methods of Biochemical Analysis*, vol. 2, p. 359. Ed. by Glick, D. New York: Interscience Publishers Inc.
Geschwind, I. I. & Li, C. H. (1957). *Biochim. biophys. Acta*, **25**, 171.
Guttmann, St. & Boissonnas, R. A. (1958). *Helv. chim. acta*, **41**, 1852.
Harris, J. I. (1958). In *Symposium on Protein Structure*, p. 333. Ed. by Neuberger, A. London: Methuen and Co. Ltd.
Harris, J. I. (1959). *Biochem. J.* **71**, 451.
Harris, J. I. & Lerner, A. B. (1957). *Nature, Lond.*, **179**, 1346.
Harris, J. I. & Li, C. H. (1954). *J. Amer. chem. Soc.* **76**, 3607.
Harris, J. I. & Roos, P. (1959). *Biochem. J.* **71**, 434, 445.
Hirs, C. H. W., Moore, S. & Stein, W. H. (1953). *J. biol. Chem.* **200**, 493.
Landgrebe, F. W. & Mitchell, G. M. (1954). *Quart. J. exp. Physiol.* **39**, 11.
Landgrebe, F. W. & Waring, H. (1950). In *Hormone Assay*, p. 141. Ed. by Emmens, C. W. New York: Academic Press Inc.
Li, C. H. (1957). *Advanc. Protein Chem.* **12**, 269.
Li, C. H., Geschwind, I. I., Dixon, J. S., Levy, A. L. & Harris, J. I. (1955). *J. biol. Chem.* **213**, 171.
Lock, J. A. (1952). *Lancet*, ii, 147.
Michl, H. (1951). *Mh. Chem.* **82**, 489.
Northrop, J. H. & Kunitz, M. (1936). In *Handbuch der biologischen Arbeitsmethoden*, Abt. 4, Teil 2, p. 2213. Ed. by Abderhalden, E. Berlin: Urban und Schwarzenberg.
Redfield, R. R. & Anfinsen, C. B. (1956). *J. biol. Chem.* **221**, 385.
Sayers, M. A., Sayers, G. & Woodbury, L. A. (1948). *Endocrinology*, **42**, 379.
Sheehan, J. C., Goodman, M. & Hess, G. P. (1956). *J. Amer. chem. Soc.* **78**, 1367.
Shepherd, R. G., Howard, K. S., Bell, P. H., Cacciola, A. R., Child, R. G., Davies, M. C., English, J. P., Finn, B. M., Meisenhelder, J. H., Moyer, A. W. & van der Scheer, J. (1956). *J. Amer. chem. Soc.* **78**, 5051.
Shepherd, R. G., Willson, S. D., Howard, K. S., Bell, P. H., Davies, D. S., Davis, S. B., Eigner, E. A. & Shakespeare, N. E. (1956). *J. Amer. chem. Soc.* **78**, 5067.
Shizume, K., Lerner, A. B. & Fitzpatrick, T. B. (1954). *Endocrinology*, **54**, 553.
Steelman, S. L., Andersen, R. N. & McGregor, R. M. (1959). *Biochim. biophys. Acta*, **33**, 256.
Synge, R. L. M. (1939). *Biochem. J.* **33**, 1924.
Thompson, R. E. & Fisher, J. D. (1953). *Endocrinology*, **52**, 496.
Waley, S. G. & Watson, J. (1953). *Biochem. J.* **55**, 328.
White, W. F. (1955). *J. Amer. chem. Soc.* **77**, 4691.

Biochem. J. (1960) **75**, 328

The Preparation and Purification of Tritium-Labelled Hexoestrol of Very High Specific Activity on the 5 mg. Scale

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Tritium can be introduced into organic compounds by catalyst-induced exchange with ionic tritium, which is usually contained in the solvent (water, acetic acid, sulphuric acid, etc.) in which the reaction takes place, by radiation-induced exchange with tritium gas (Wilzbach, 1957), or by catalytic addition of tritium to unsaturated compounds. Exchange with ionic tritium suffers from the disadvantages that the high temperatures required may cause decomposition (Bloch & Rittenberg, 1943), that specific activities are limited by that obtainable in the solvent, which

must be used in large excess, and that exchange may not be complete within a reasonable time. Exposure of organic compounds to tritium gas results in exchange with little decomposition even of very labile compounds but yields products of only low specific activities. Provided that a suitable unsaturated parent compound can be obtained, catalytic addition of tritium is undoubtedly the best method, both because of the high specific activities which are theoretically obtainable and because of the relatively mild conditions of the reaction.

Because the specific activities obtainable with tritium-labelled compounds are always high compared with those of ^{14}C-labelled compounds and because of its low cost the use of tritium is becoming more common, and some tritium-labelled compounds are available commercially. Many of the methods of preparation (e.g. those of Done & Payne, 1956; Crawhall & Smyth, 1958; Berstein, Bennett & Fields, 1952) are carried out on the conventional scale for hydrogenation (5–10 m-moles). This almost always involves the dilution of the tritium with many times its own volume of hydrogen since, when the weight of compound being hydrogenated is equivalent to 100 ml. or more of hydrogen, it is scarcely feasible to use tritium at its maximum specific activity (97 % $^3H_2 \equiv 2{\cdot}7$ c/ml. at s.t.p.). In work with compounds of high specific activity the weight of compound required is usually small and hence its preparation on a micro-scale is suitable.

The highest specific activities so far reported seem to be those of the labelled oestrogens prepared by O'Donnell & Pearlman (1958), who hydrogenated 26·8 mg. of Δ^6-dehydro-oestrone acetate with 3 c of tritium and obtained oestrone acetate of specific activity 17·5 mc/mg. in 16 % radiochemical yield. The apparatus is not described in this preliminary communication but was clearly not on the conventional scale.

Dodds, Golberg, Lawson & Robinson (1939) reported the hydrogenation of 3:4-di-(4-hydroxyphenyl)hexa-2:4-diene (dienoestrol) to *meso*-3:4-di-(4-hydroxyphenyl)hexane (hexoestrol) in good yield over a palladium-black catalyst. Williams & Ronzio (1950), working on the conventional scale with tritium–hydrogen of low specific activity, used the same reaction in their preparation of tritium-labelled hexoestrol. They isolated both hexoestrol and *iso*hexoestrol (the racemic compound) from their reaction mixture by crystallization procedures, but in all experiments obtained a yield of considerably more than 100 %, which they thought might be due to solvent of crystallization.

For several years we have had in use in our laboratories a hydrogenation apparatus which works on the 50 μmoles scale ($\equiv$ 1 ml. of hydrogen at s.t.p.), in which it is therefore feasible to use tritium of maximum specific activity. It has been used for the preparation, from a nominal 10 % tritium–hydrogen mixture, of labelled hexoestrol and stearic acid. Biochemical experiments with these compounds have been reported (Dodds, Folley, Glascock & Lawson, 1958; Glascock & Hoekstra, 1959; Glascock & Reinius, 1956; Glascock, Duncombe & Reinius, 1956). Labelled hexoestrol prepared from 97 % tritium has also been prepared and is now being used.

A preliminary description of an early version of the apparatus has been published (Glascock & Reinius, 1954) and has been quoted (Glascock, 1954). Because of improvements in the apparatus and in the technique of purifying the tritiated hexoestrol a full description of the method is now presented.

It was to be expected that, when preparations on the 5 mg. scale were carried out, the separation of the isomers by crystallization as used by Williams & Ronzio (1950) would be difficult; indeed such attempts to isolate hexoestrol from the products of the hydrogenation of dienoestrol on this scale were unsuccessful. A paper-chromatographic method for the quantitative separation of hexoestrol from *iso*hexoestrol was therefore developed with artificial mixtures of the isomers, and was then successfully applied to the products of hydrogenation of dienoestrol with hydrogen or tritium.

As the reaction may not always go quite to completion a small amount of unchanged dienoestrol may be present in the reduction products and cannot be separated from hexoestrol by the chromatographic method. A spectrophotometric method was therefore devised which enabled dienoestrol in mixtures of hexoestrol and *iso*hexoestrol to be detected (to a lower limit of 2 %) and reaction products were tested for dienoestrol before isolation of the hexoestrol was begun.

MATERIALS AND ANALYTICAL METHODS

Materials

Dienoestrol was obtained from L. Light and Co. Ltd., Colnbrook, Bucks, and recrystallized from aq. ethanol shortly before use, m.p. 227–228°. Dioxan was purified by treatment with HCl and then with KOH solution, followed by drying and redistillation as described by Vogel (1957). Palladium-black catalyst was prepared according to the method of Heilbron, Sexton & Spring (1929). Hydrogen gas, as supplied in cylinders by British Oxygen Gases Ltd., was purified by passage through a U-tube containing granular charcoal, previously heated to expel dissolved gas in a stream of H_2, and cooled in liquid air. Commercial 95 % ethanol (James Burrough Ltd.) was used in the spectrophotometric work. Benzene was the pure, crystallizable grade; light petroleum, ethyl acetate and methanol were laboratory-reagent grades and diethyl ether was the

anaesthetic grade (all supplied by May and Baker Ltd.). The B (3) solvent of Bush (1952) used for the column-partition chromatography was first made from these solvents and the upper phase was then redistilled to remove traces of non-volatile matter. Inorganic salts were of laboratory-reagent grade and the compound 4-aminophenyl 2-diethylaminoethyl sulphone was supplied by L. Light and Co. Ltd. The diazonium-salt reagent was prepared by diazotizing this compound as described by Boscott (1951).

Celite 545, used for partition chromatography, was obtained from Johns–Manville and Co. Ltd. It was prepared for use by heating in air at 600° for 16 hr. and then extracting in a Soxhlet apparatus first with benzene and then with methanol.

Alumina-impregnated paper. This was prepared by a method based on the work of Datta & Overell (1949), Datta, Overell & Stack-Dunne (1949) and Bush (1953). Eight Whatman 3 MM papers (46 cm. × 18 cm.) were dipped in filtered aqueous 6·5% (w/v) $Al_2(SO_4)_3,12H_2O$, removed immediately and hung for 10 min. to drain in chromatography tanks, four papers to each tank. Sufficient aqueous NH_3 soln. (sp.gr. 0·880) was then added by means of a long-stemmed polythene funnel to cover the floor of each tank to a depth of about 2 cm. The tanks were kept closed for 24 hr., after which the NH_3 was sucked out and the papers were washed by passing tap water (filtered through glass wool to remove suspended matter) to the bottom of each tank and allowing it to overflow. Washing was continued for 24 hr. and the papers were dried in a well-ventilated room maintained at 28°. They were dry to the touch in 30 min. In order to prepare papers of constant adsorptive properties it was found to be particularly important to standardize the drying conditions: papers hung in the laboratory at 20–25° took more than 2 hr. to dry and these had an inconveniently high adsorptive power. The properties of the impregnated papers could be tested by chromatographing, as described later, 10 μg. of a mixture of hexoestrol and *iso*hexoestrol on a test strip 2 cm. wide cut from the long edge of each. It was found that papers, when stored at 28° in the dark, retained their satisfactory properties for at least 1 year.

Analytical methods

Radioautographs. The positions of radioactive spots could be found by placing the paper in contact with Ilford Industrial G X-ray film. Times of exposure varied from 15 min. (when tritium of 97% purity had been used in the preparation) to several hours.

Measurement of radioactivity. The specific activity of the tritiated hexoestrol was determined either by liquid-scintillation counting or by gas counting. For scintillation counting, a portion of a suitable subdilution in toluene was added to a solution in toluene of 2:5-diphenyloxazole (4 g./l.) and counted in an Ekco N 612 scintillation counter cooled to −18°. For gas counting a portion of a suitable subdilution was added to a weighed amount of hexoestrol dissolved in ether. The ether was distilled off, and the residue dried to a constant weight and submitted to combustion and gas counting as described by Glascock (1954).

Spectrophotometric estimation of dienoestrol in mixtures with hexoestrol and isohexoestrol. Solutions were made up in 95% ethanol and measurements of extinction were made with a Unicam SP. 500 spectrophotometer with 1 cm. silica cells.

The light-absorption curves for hexoestrol and *iso*hexoestrol each at a concentration of 50 μg./ml. were determined over the range 235–265 mμ and found to be almost identical, each with a very low minimum at 247 mμ, whereas dienoestrol at a concentration of only 5 μg./ml. had much greater absorption, decreasing steadily in the range 235–265 mμ. The extinctions at 247 mμ for these hexoestrol, *iso*hexoestrol and dienoestrol solutions were respectively 0·035, 0·02 and 0·27. When E at 247 mμ for six concentrations of dienoestrol in the range 1–10 μg./ml. was plotted against concentration a straight line was obtained, indicating that Beer's law was obeyed. It thus seemed probable that measurement of E at 247 mμ could provide a simple method for the estimation of dienoestrol when mixed with hexoestrol and *iso*hexoestrol, and that in a total concentration of 50 μg./ml. the presence of as little as 1 μg. of dienoestrol/ml. (2% of total solute) could be detected.

To test this, the absorption curve of a solution containing hexoestrol (50 μg./ml.) and dienoestrol (2·5 μg./ml.; dienoestrol 4·8% of total solute) was compared with that of a hexoestrol solution (50 μg./ml.) over the range 235–265 mμ. The difference in E at 247 mμ of the two solutions indicated, from the extinction–concentration line for dienoestrol, the presence of 2·4 μg. of dienoestrol/ml. (4·6% of total solute). This and similar tests with dienoestrol, hexoestrol and *iso*hexoestrol mixtures confirmed that the method was capable of estimating unchanged dienoestrol in reduction products in proportions as low as 5%, with an error not greater than ±10%, and of reliably detecting as little as 2%. It was considered that unchanged dienoestrol in a proportion of 5% or less in reduction products would not interfere with the efficient isolation of the hexoestrol.

Melting point of hexoestrol. All melting points were determined on a Kofler heating-stage microscope RCH, the crystals being crushed beneath a cover slip. This is particularly important for hexoestrol, which sublimes rapidly above 150°.

We noticed that specimens of hexoestrol prepared in our apparatus from dienoestrol with either hydrogen or hydrogen containing 10% of tritium did not melt completely at 186°. A proportion estimated at 5–10%, visible through the analyser and polarizer in the crossed position, did not melt below a temperature of about 190°. It was feared that this indicated an impurity, and to examine this possibility several reference specimens of hexoestrol were tested. These comprised several commercial specimens as received, a specimen recrystallized from benzene, another recovered after slow sublimation at 110–115° and a specimen which had been recrystallized from ethanol containing a little HCl to remove any traces of alkali-metal salts present (this specimen was kindly supplied by Dr D. A. Peake of Boots Pure Drug Co. Ltd.).

All specimens showed identical behaviour at their melting points and it is concluded that this behaviour is not due to an impurity but is characteristic of pure hexoestrol, especially since it is known that most of the specimens of commercial origin had been synthesized by a method other than the reduction of dienoestrol (Dr D. A. Peake, personal communication).

Chromatography (analytical). It was found that the isomers of hexoestrol could not be separated on Whatman no. 2 paper by the solvent systems of Bush (1952). They

could be separated, however, on alumina-impregnated paper by the method which he described for the separation of steroid oestrogens.

Spots containing 5 and 10 μg. of the hydrogenation product and 5 and 10 μg. of a reference mixture (1:1) of hexoestrol and *iso*hexoestrol were deposited at well-separated points along a base line 8 cm. from the end of a sheet of alumina-impregnated paper. It was important to use benzene for applying these compounds to the paper because polar solvents caused some irreversible adsorption. The chromatogram was then developed with 8% (v/v) of ethyl acetate in benzene in a chromatography tank, on the floor of which was a layer of the same solvent mixture to maintain the atmosphere saturated with vapour. The solvent front took 2–3 hr. to reach the end of the paper, which was then air-dried and sprayed with aqueous 5% K_2CO_3 and then with diazonium-salt reagent. If the papers had been properly prepared the position of the isomers in the reference mixture was revealed as pink spots at R_F 0·6 (hexoestrol) and 0·35 (*iso*hexoestrol). Hydrogenation products, known from spectrophotometric analysis to be free from dienoestrol, gave two spots of the same R_F and colour as the spots of the reference mixture. Proof of the identity of the faster-running compound followed from its isolation described below. One would, of course, expect the other compound to be *iso*hexoestrol, and although this was not proved by isolation the fact that the light-absorption curves for hydrogenation products and the standard hexoestrol–*iso*hexoestrol mixture were the same was good evidence for its identity.

By visual comparison of the azo-dye spots given by the reference mixture and by samples of hydrogenation products it was estimated that the products contained 50–70% of hexoestrol and 30–50% of *iso*hexoestrol. Although a closer estimate than is represented by this range was not possible visually we are convinced that the content of *iso*-hexoestrol was never less than 30%.

Other experiments showed that the minimum quantity of either isomer detectable by chromatography and the diazonium-salt reagent was about 1 μg.; much smaller quantities of labelled compounds could be detected by long exposure to X-ray film.

METHOD OF PREPARATION

Hydrogenation apparatus

The high-vacuum apparatus used is shown in Fig. 1. It was evacuated to a pressure of 10^{-6} mm. Hg or less by means of a mercury-vapour pump suitably backed by a mechanical pump. T_1 and T_2 were Toepler pumps with bulbs of capacity about 300 ml. The tubes below stopcocks 10 and 24 were calibrated as for a McLeod gauge (Glascock, 1954) and were of such a size that they were capable of measuring up to about 1·25 ml. of gas at s.t.p.

Hydrogenations took place in small flasks F_1 and F_2, of capacity 0·4–0·5 ml., attached to the apparatus by means of a standard B5 ground-glass joint. The part of the apparatus to which the flask F_1 was attached is shown in greater detail in Fig. 2. The corresponding part of the apparatus to which F_2 was attached was exactly similar. The manifold bounded by stopcocks 8 and 10, by the valve V_1 (see below) and F_1 was made of capillary tubing (internal diam. 2 mm.). The capacity of the manifold + flask F_1 was thus about 1·5 ml.

The bulbs of the device R_1 were of about 10 ml. capacity. It was charged with mercury so that by evacuating, or admitting air to, the bulb beneath stopcock 6 the other bulb was either emptied or filled (under the control of stopcock 7), the small glass valve V_1 preventing accidental overshooting of mercury into the manifold above it. The device was used to compress the charge of tritium–hydrogen to about 1 atm., as will be described later.

S was a tube containing purified dioxan in contact with several pieces of bright sodium. When the dioxan was first put into the tube it was frozen in liquid air and the space above it evacuated through stopcocks 31, 32 and 33. This process removed all the dissolved gas. The dioxan was then thawed and refrozen and the gas pumped off; this cycle was repeated several times. The bulb above the tube S had a capacity of 250 ml. and when filled with dioxan vapour in equilibrium with liquid at laboratory temperature (21°) it therefore contained an amount equivalent to about 50 μl. of liquid, the volume used as a solvent for each hydrogenation.

The ampoule of tritium G was sealed to the apparatus below stopcock 19, a side arm being provided to hold the mild-steel weight with which the break-seal was opened. The space between stopcocks 18 and 19 had a capacity of about one-twentieth of the total space beneath stopcock 19 and could thus be used as a gas pipette for the withdrawal of small amounts of tritium.

P was a measuring device for taking any desired volume of gas up to the capacity of the Toepler pumps and was used, for example, when the tritium already withdrawn from G measured and transferred to the reaction vessel (F_1 or F_2) had to be diluted with H_2 to the volume required for the hydrogenation. The capacity of space x between stopcocks 16 and 17 was 0·25–0·35 ml. and that of space y beneath stopcock 17 was 1·0–2·0 ml. Gas was allowed to enter P and the manifold above it to a pressure p recorded on the manometer M. Stopcocks 16 and 17 were closed and the gas remaining in the apparatus was pumped away. The gas in space x was allowed to expand into either Toepler pump and the gas trapped by the rising mercury when the pump was operated in the usual way was measured. The gas in y was treated in a similar way. These volumes were proportional to the pressure p at which the gas was originally admitted to P and, after determining the calibration constants for both x and y with reference to both Toepler pumps, any volume of gas could be

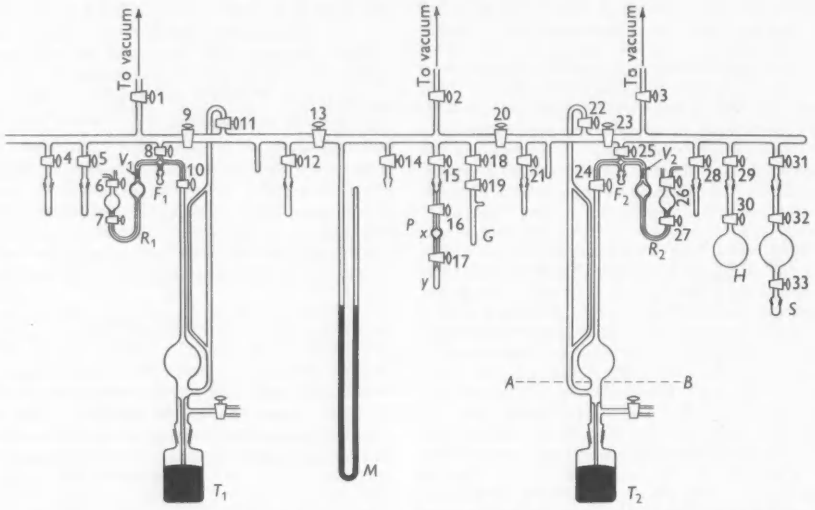

Fig. 1. High-vacuum apparatus for hydrogenation on the scale of 50 μmoles. 1–33, Stopcocks; T_1 and T_2, Toepler pumps; M, mercury manometer; R_1 and R_2, devices for temporarily increasing the volume of reaction-flask manifold (see text); F_1 and F_2, reaction flasks; P, gas-measuring device (see text); G, ampoule of tritium; S, solvent reservoir; H, reservoir of purified hydrogen.

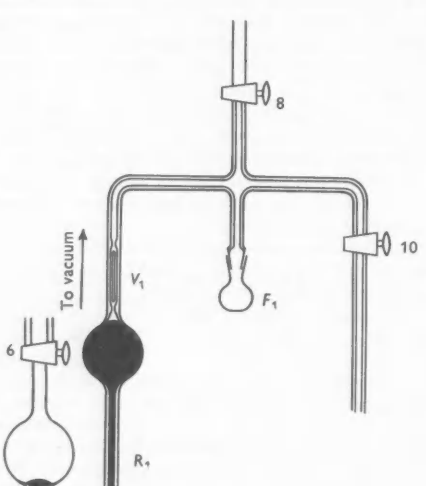

Fig. 2. Device R_1 of Fig. 1 in greater detail. F_1, Reaction flask; V_1, glass valve to prevent overshooting of mercury into manifold above.

taken simply by adjusting the pressure at which gas was admitted to P, which could be done very accurately. In our apparatus the constants were 2·5 and 17·5 μl./cm. Hg pressure for T_1 and of similar values for the other Toepler pump T_2.

Hydrogenation procedure

About 5 mg. of dienoestrol and 2 mg. of palladium black were accurately weighed into a reaction flask containing a piece of soft-iron wire (about 2 mm. long) encased in glass, which served as a magnetic stirrer. The flask was attached to the manifold at F_2, the joint being lubricated with silicone grease. It was evacuated through stopcock 25, care being taken to avoid withdrawing the air too quickly otherwise rapid desorption from the catalyst might scatter it about the apparatus. The bulb above the solvent reservoir S was evacuated, stopcock 32 closed and stopcock 33 left open for a few minutes to allow the bulb to fill with vapour. The dioxan vapour in the bulb was then condensed into the flask F_2, which was chilled in liquid air, by way of stopcock 25. Tritium was withdrawn from the ampoule and pumped past stopcock 24 into the reaction flask and manifold. In order for this to be possible the mercury had to be withdrawn from the upper bulb of R_2, which was refilled with mercury after the gas has been transferred. This was always necessary when all but the smallest volumes of gas were compressed past stopcock 24.

The volume of tritium transferred at each stroke was noted and, if necessary, the volume was made up to the calculated value with H_2 measured in the device P as already described. The calculated volume depended on the

weight of dienoestrol and the blank value for catalyst and solvent. This blank was the volume of H_2 taken up irreversibly by a weighed amount of catalyst in 50 μl. of dioxan. It amounted to 20 μl./mg. of catalyst in our apparatus and with our materials. It was best to use about a 5% excess of total H_2 for the hydrogenation of dienoestrol in order to drive the reaction as near to completion as possible.

The initial volume of tritium–hydrogen was checked by allowing it to expand past stopcock 24 into the bulb of the Toepler pump with the mercury set at the level *A–B*, and remeasuring in the usual way. It was returned to the reaction flask and manifold, the liquid air was removed from around the reaction flask and the magnetic stirrer was started. The course of the reaction could be followed at timed intervals by removing the stirrer motor, chilling the reaction flask in liquid air and measuring the unreacted gas as described above. Uptake ceased in from 3 to 5 hr. when, allowing for catalyst blank, 98–102% of the theoretical volume of gas had been taken up.

At the end of the reaction the residual gas was either discarded or, by means of the other Toepler pump, transferred to a break-seal tube attached in the place of F_1. The solvent dioxan was distilled into a tube below stopcock 28 and the small amount of gas which was released was measured in the Toepler pump and taken into account when calculating net uptake.

Isolation procedure

The contents of the reaction flask were extracted with 95% ethanol and the solution was filtered. A solution of concentration 50 μg./ml. in 95% ethanol was then prepared on the assumption that the theoretical weight of hydrogenation products had been formed and that all had been extracted. From the extinction of this solution the dienoestrol content was determined and always found to be less than 5% when the theoretical volume of hydrogen had been taken up.

The specific activity of the product was also determined at this stage. To 100 mg. of carrier hexoestrol dissolved in ether a measured volume of a subdilution of the extract was added such that the carrier would acquire a convenient specific activity. The solution was then evaporated to dryness, the residue dissolved in dilute alkali (to displace any tritium on the phenolic groups), precipitated with acid, extracted with ether and isolated again. Its specific activity was then measured and the specific activity of the original mixed product, free from tritium in the phenolic groups, was calculated. This value was needed at a later stage in the purification when the concentration of hexoestrol in a benzene solution was calculated from the specific activity of the solution.

The main ethanolic solution was then reduced in volume to about 1 ml. by evaporation in a stream of N_2, 0·1 ml. of N-KOH was added and the solution was diluted to 10 ml. with water. The pH was brought to 7·0 by the addition of dilute HCl and the solution was extracted with 3 × 10 ml. of benzene–ether (4:1). The combined extracts were washed with 3 × 5 ml. of water and then distilled until the volume of the solution was 2 ml. This removed both water and ether and left the hydrogenated product dissolved only in benzene.

The chromatographic separation was extended to the preparative scale. Half of the solution in 2 ml. of benzene was applied to each of two impregnated papers along the base line. By applying the solution to the paper very slowly in a current of warm air from a pipette with its delivery end bent at a right angle the solute was deposited in a band about 1 cm. wide. The two sheets of paper were then eluted with 8% (v/v) of ethyl acetate in benzene by the descending method until the solvent front reached nearly to the end of the paper. The papers were dried at room temperature and radioautographs were prepared. The time of exposure depended, of course, upon the total radioactivity on the paper but with hexoestrol prepared from 10% tritium about 4 hr. was required to give sufficient blackening of the film. The position of the two isomers was then revealed as two bands across the film each about 4 cm. wide separated by a white zone of about the same width. The part of each chromatogram corresponding to the leading band was then cut out and the two rectangular strips (each about 5 cm. × 15 cm.) were pinned to a piece of unused alumina-impregnated paper and eluted overnight with the ethyl acetate–benzene solvent mixture. About 10 ml. of solvent passed through each of the strips, thus extracting all the hexoestrol, and was collected in a vessel standing on the floor of the tank. Because chemical attack ultimately due to radiation is least extensive in benzene solution the solvent mixture used for elution was distilled off and the residue dissolved in pure dry benzene. When tritium of the highest available specific activity had been used in the preparation the labelled hexoestrol was never isolated in the solid state at all because of radiolysis (see below). In these preparations the two strips of paper were eluted with pure benzene. Hexoestrol moves very slowly in this solvent but nevertheless about 90% was extracted overnight and most of the remainder within a further 24 hr. About one-third of the benzene was then distilled off to remove traces of water, the residual solution assayed for radioactivity and then made up to a volume corresponding to a convenient hexoestrol concentration (usually 50 μg./ml.).

When a spot containing 60 μg. of labelled hexoestrol separated in this way was chromatographed on alumina-impregnated paper no *iso*hexoestrol was detected either by the diazonium reagent or by radioautography. And from the appearance of the spots produced by 2 and 5 μg. samples of authentic *iso*hexoestrol run at the same time it was concluded that the labelled hexoestrol contained less than 2% of *iso*hexoestrol.

Even so, recrystallization, after removal of the benzene, did not give the hexoestrol in good yield with the correct sharp melting point. This was attributed to the high losses involved in recrystallization on the 2–3 mg. scale, to traces of foreign matter extracted from the chromatography paper and to traces of radiation-induced decomposition products. Further purification on a partition column was therefore used. A tube 1 cm. in diameter was packed as described by Martin (1949) with 10 g. of Celite 545 mixed with 5 g. of the lower phase of the solvent system of Bush (1952) (light petroleum–benzene–methanol–water; 10:5:12:3, by vol.). The column was first washed with 200 ml. of redistilled upper phase to remove soluble impurities. The benzene solution of labelled hexoestrol was evaporated to dryness and the residue taken up in a combination of 3 ml. of upper phase with 1 ml. of lower phase. It was then added to the top of the column, which was operated in the usual way at a flow rate of about 15 ml./hr., and 15 ml. fractions were collected. In an actual run with

hexoestrol, prepared from tritium, of nominal specific activity 330 mc/ml., and 5·5 mg. of dienoestrol, fractions 8, 9, 10 and 11 all gave crystalline residues together weighing 2·8 mg., m.p. 186–188° undepressed on admixture with authentic hexoestrol. Yield 51%. This preparation had a specific activity of 13·7 mc/mg. After being stored in a desiccator for 2 weeks its melting point diminished by 10° because of radiolysis. Another preparation, made from tritium of nominal specific activity 2·5 mc/μl. (97% tritium) had a specific activity of 118 mc/mg. It was not further purified on the partition column because that would have involved evaporation of the benzene solution to dryness and we have not demonstrated that the column would remove the small quantities of radiolytic products that might form before the dry residue could be taken up into solution again. The impurities derived from the paper in no way impair the usefulness of the product for biological purposes as they are not labelled and are most unlikely to be oestrogenic.

The specific activity of these preparations was only about 25% of that expected if the tritium used had its nominal specific activity.

DISCUSSION

The preparation of tritium-labelled hexoestrol on this scale proceeded much as was expected from previously published work although the yield was less than was expected. Dodds *et al.* (1939) reported a nearly quantitative yield of hexoestrol when dienoestrol was hydrogenated over a palladium-black catalyst, whereas substantial amounts of *iso*hexoestrol are formed under the conditions of our work. This is shown not only by the amount of hexoestrol actually isolated but also by the amount of *iso*hexoestrol revealed by chromatography. Williams & Ronzio (1950) reported a yield of at least 25% of *iso*hexoestrol in their preparations but their observations may not be quite relevant because they used a different catalyst (palladium on charcoal). Moreover they separated the isomers by recrystallization, which is less nearly quantitative than chromatography and they had difficulty, as already noted, with total yields of greater than 100%.

Williams & Ronzio (1950) also reported that their reaction went more quickly with hydrogen containing tritium than with hydrogen alone. Their reaction was carried out in ethanol or acetone with activities of tritium which gave a product of specific activity 1 mc/mg. With levels of radioactivity from 10 to 100 times as great as theirs we have not observed that tritium increases the rate of the reaction in dioxan solution. Indeed with high activities of tritium (10% or more) we have observed the reverse effect: unless the reaction goes to completion within a few hours it may come to a standstill. The magnetic stirrer, which was not originally used (Glascock & Reinius, 1954), was introduced into the technique for this reason. We at first thought that the apparent inhibitory effect of tritium might be due to some catalyst poisons contained in it but since careful purification over charcoal chilled in liquid air made no difference we now provisionally attribute the inhibitory effect to the formation of catalyst poisons by radiolysis during the course of the reaction.

Our method also differs from those carried out on the conventional scale in the relatively large amount of catalyst used. The weight of palladium is about 40% of that of the dienoestrol instead of 1–2% as would normally be used. With smaller amounts of catalyst the reaction goes too slowly even with pure hydrogen. This is probably connected with the fact that the pressure of the hydrogen is not constant but diminishes as the reaction proceeds and finishes at only about 5% of the initial pressure.

Martin (1949), in his theoretical treatment of partition chromatography, predicted that it would in general be less successful than the adsorption method in separating isomers and our results are in accord with this theory. Alumina-impregnated paper is thought to be of possible value in dealing with some separation problems where paper-partition chromatography has failed; it has an advantage over adsorption columns in that the same paper may be used for microgram-scale analysis and thus an evaluation of its possible usefulness quickly and easily made. When it is used on a preparative scale the introduction of traces of foreign matter into the compound being isolated is probably unavoidable and the use of a Celite partition column for final purification is particularly to be recommended as the Celite and solvents are capable of being very highly purified.

SUMMARY

1. An apparatus for the catalytic hydrogenation of unsaturated organic compounds on the scale of 50 μmoles is described. It is used for the hydrogenation of dienoestrol with hydrogen containing up to 97% of tritium, when a product of specific activity 118 mc/mg. is obtained.

2. A method for the separation of the isomeric products hexoestrol and *iso*hexoestrol on alumina-impregnated paper is given together with a column-partition method for the further purification of the separated hexoestrol from unlabelled impurities derived from the paper.

3. A spectrophotometric method for the detection of unchanged dienoestrol in the reduction products to a lower limit of 2% is also described.

4. Hydrogen uptake usually ceases within a few hours, when it is found that the theoretical volume of hydrogen ($\pm$2%) has been taken up and less than 2% of unchanged dienoestrol remains in the reaction mixture.

REFERENCES

Berstein, I. A., Bennett, W. & Fields, M. (1952). *J. Amer. chem. Soc.* **74**, 5763.
Bloch, K. & Rittenberg, D. (1943). *J. biol. Chem.* **149**, 505.
Boscott, R. J. (1951). *J. Endocrin.* **7**, 154.
Bush. I. E. (1950). *Nature, Lond.*, **166**, 445.
Bush, I. E. (1952). *Biochem. J.* **50**, 370.
Crawhall, J. C. & Smyth, D. G. (1958). *Biochem. J.* **69**, 280.
Datta, S. P. & Overell, B. G. (1949). *Biochem. J.* **44**, xliii.
Datta, S. P., Overell, B. G. & Stack-Dunne, M. (1949). *Nature, Lond.*, **164**, 673.
Dodds, E. C., Folley, S. J., Glascock, R. F. & Lawson, W. (1958). *Biochem. J.* **68**, 161.
Dodds, E. C., Golberg, L., Lawson, W. & Robinson, Sir Robert (1939). *Proc. Roy. Soc.* B, **127**, 140.
Done, J. & Payne, P. R. (1956). *Biochem. J.* **64**, 266.
Glascock, R. F. (1954). *Isotopic Gas Analysis for Biochemists*, p. 180. New York: Academic Press Inc.
Glascock, R. F., Duncombe, W. G. & Reinius, L. R. (1956). *Biochem. J.* **62**, 535.
Glascock, R. F. & Hoekstra, W. G. (1959). *Biochem. J.* **72**, 673.
Glascock, R. F. & Reinius, L. R. (1954). In *Radioisotope Conference*, vol. 1, p. 262. Ed. by Johnston, J. E., Faires, R. A. & Millett, R. J. London: Butterworths Scientific Publications Ltd.
Glascock, R. F. & Reinius, L. R. (1956). *Biochem. J.* **62**, 529.
Heilbron, I. M., Sexton, W. A. & Spring, F. S. (1929). *J. chem. Soc.* p. 926.
Martin, A. J. P. (1949). *Symp. biochem. Soc.* **3**, 11.
O'Donnell, V. J. & Pearlman, W. H. (1958). *Biochem. J.* **69**, 38P.
Vogel, A. I. (1957). *Text-book of Practical Organic Chemistry*, 3rd ed. p. 177. London: Longmans Green and Co.
Williams, D. L. & Ronzio, A. R. (1950). *J. Amer. chem. Soc.* **72**, 5787.
Wilzbach, K. E. (1957). *J. Amer. chem. Soc.* **79**, 1013.

Biochem. J. (1960) **75**, 335

Fluorescence-Polarization Spectrum and Electronic-Energy Transfer in Tyrosine, Tryptophan and Related Compounds

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The polarization spectrum of the fluorescence is the set of values of the polarization of the fluorescence emitted upon excitation with light of different wavelengths. Simple symmetry relations exist among the polarizations of the fluorescence emitted in different directions, and by excitation with light of different states of polarization (Perrin, 1929; Soleillet, 1929), so that the values for a single direction of observation and a given state of polarization of the exciting radiation contain all the relevant information. The data recorded in this paper are for the fluorescence emitted at right angles to the direction of the exciting, unpolarized beam.

The absolute value of the polarization of the fluorescence emitted by molecules in solution can decrease as a result of the Brownian molecular rotations (Perrin, 1926), as well as by intermolecular transfer of the excited state (Sveshnikoff & Feofilov, 1940; Weber, 1954). In a dilute solution in which the average distance between molecules is much greater than the distance at which transfer can readily take place (of the order of 30 Å), and in a medium of high viscosity in which the rotational relaxation time of the molecules is much longer than the lifetime of the excited state, both these causes of depolarization disappear and the polarization reaches a maximum absolute value, the principal polarization. In some well-studied cases this appears still inferior to the value that would be expected from a knowledge of the relative orientation of the absorption and emission oscillators, due to the molecular-torsional vibrations (Jablonski, 1950), and perhaps to other causes at present imperfectly understood. A plot of the principal polarization against wavelength, the principal polarization spectrum, reflects to a first approximation the relative orientations of the transition moments associated with the absorption and emission of light (Jablonski, 1935). These directions of the transition moments are expected to remain constant for each electronic transition. Ideally, the principal polarization spectrum should consist of regions of constant polarization where the excitation falls upon one electronic band alone, joined by regions of rapidly changing polarization where there is overlap of contiguous electronic bands. A study of the principal polarization spectrum should lead to the enumeration of the existing electronic bands, and to the distinction between

vibrational details inside an electronic band and two or more overlapping electronic transitions. In the latter case it should be possible to determine the importance of each of the contributing electronic transitions on changing the environment or in comparing related substances, a possibility that is not offered by a study of the absorption spectrum alone.

THEORETICAL

The intermolecular transfer of the electronic energy has been followed by observations on the polarization of the fluorescence of concentrated solutions in the transfer among like molecules (phenol–phenol and indole–indole), and by observations of the fluorescence spectrum of mixtures in the transfer among different molecules (phenol–indole).

The transfer of electronic energy among molecules in solutions is now well established. The theories developed (Perrin, 1932; Förster, 1947; Oppenheimer & Arnold, 1950) aim at calculating the characteristic distance R between two molecules at which the chances of transfer and of emission without transfer are equal. In Förster's theory R can be calculated explicitly from the maximum molar absorption coefficients E_1 and E_2 of the longest wavelength-absorption band of the donor and acceptor molecules respectively, the refractive index of the medium n, the wave-number maxima of the emission of the donor ν_e and of absorption of the acceptor ν_a, and the overlap integral J_ν:

$$J_\nu = \int_0^\infty \epsilon(\nu)\, F(\nu)\, d\nu. \qquad (1)$$

In the last equation $\epsilon(\nu)$ is the molar absorption coefficient of the acceptor and $F(\nu)$ the molar emission coefficient of the donor. The molar emission function $F(\nu)$ represents the photon-spectral distribution of the fluorescence in units set by the equality

$$F(\nu_e) = E_2,$$

J_ν can be determined by graphical integration, but it is simpler, and sufficiently accurate for our purpose, to calculate it on the assumption that the absorption and emission bands are represented by Gaussian distributions with standard deviations σ_a and σ_e:

$$\epsilon(\nu) = E_1 \exp -\left(\frac{\nu-\nu_a}{\sigma_a}\right)^2, \qquad (2)$$

$$F(\nu) = E_2 \exp -\left(\frac{\nu-\nu_e}{\sigma_e}\right)^2. \qquad (3)$$

σ_a and σ_e are calculated from the data referring to the overlapping sides of the bands. It is then immaterial whether they represent accurately the opposite side since the overlap is here identically zero. With the Gaussian approximations,

$$J_\nu = E_1 E_2 \frac{\sqrt{\pi}}{\left(\frac{1}{\sigma_a^2}+\frac{1}{\sigma_e^2}\right)^{\frac{1}{2}}} \exp -\left[\frac{(\nu_a-\nu_e)^2}{\sigma_a^2+\sigma_e^2}\right]. \qquad (4)$$

According to Förster's theory,

$$R_c = \left[\frac{1660}{n^2}\cdot\frac{\tau J_\nu}{\left(\frac{\nu_a+\nu_e}{2}\right)^2}\right]^{\frac{1}{6}} 10^{-6}\ \text{cm.}, \qquad (5)$$

with τ lifetime of the excited state and n refractive index of solvent.

The calculated values given by the last equation, R_c, will be compared with the values R experimentally determined from depolarization or fluorescence spectral data. The theory of concentration depolarization has been developed elsewhere (Weber, 1954). If the reciprocal of the polarization, $1/p$, is plotted against the molar concentration c, from the slope s and the intercept $1/p_\infty$ of the straight line obtained,

$$R = \left[\frac{15s\ 10^3}{4\pi N\left(\frac{1}{p_\infty}+\frac{1}{3}\right)}\right]^{\frac{1}{6}} (2a)^{\frac{1}{2}}, \qquad (6)$$

where N is Avogadro's number and a is the average radius of the molecules. The determination of R of the phenol–indole transfer from the experimental data referring to the fluorescence spectrum of the solutions is done as follows. Let

λ = transition probability of emission of phenol,

μ_{11} = transition probability of transfer from phenol to phenol,

μ_{12} = transition probability of transfer from phenol to indole.

The probability of n transfers among phenol molecules, followed by emission by phenol, W_{n1}, equals

$$W_{n1} = \left(\frac{\mu_{11}}{\lambda+\mu_{11}+\mu_{12}}\right)^n \frac{\lambda}{\lambda+\mu_{11}+\mu_{12}},$$

and the probability of n transfers among phenol molecules followed by transfer to indole, W_{n2}

$$W_{n2} = \left(\frac{\mu_{11}}{\lambda+\mu_{11}+\mu_{12}}\right)^n \frac{\mu_{12}}{\lambda+\mu_{11}+\mu_{12}}.$$

The total emission from the excited phenol molecules is

$$W_1 = \sum_{n=0}^{n=\infty} W_{n1} = \frac{\lambda}{\lambda+\mu_{12}},$$

and the total phenol excitation transferred to indole is

$$W_2 = \sum_{n=0}^{n=\infty} W_{n2} = \frac{\mu_{12}}{\lambda+\mu_{12}}.$$

W_1 and W_2 are the fractions of the original phenol excited states appearing either as emission by the phenol or as excited states of the indole, since $W_1+W_2=1$. Also

$$\frac{W_2}{W_1}=\frac{\mu_{12}}{\lambda}. \tag{7}$$

The observables from which (μ_{12}/λ) are determined are the expected and observed ratios of the phenol fluorescence to the total fluorescence of the sample. These are respectively:

$$A_P=P_e/(P_e+I_e);\quad a_P=P/(P+I),$$

where subscript e refers to the expected fluorescences of indole (I) and phenol (P). If the fluorescence of the phenol appearing as excitation of the indole is Δ, then

$$P=P_e-\Delta,$$
$$I=I_e+\Delta(q_I/q_P),$$

where q_I and q_P are respectively the quantum yields of the fluorescence of indole and phenol and the reasonable assumption is made that both direct and indirect excitation of the indole have identical quantum yield. From the preceding equations,

$$a_P=\frac{P_e-\Delta}{P_e+I_e+\Delta\left(\frac{q_I}{q_P}-1\right)};$$

$$\frac{\Delta}{P_e+I_e}=\frac{A_P-a_P}{1+a_P\left(\frac{q_I}{q_P}-1\right)},$$

so that finally

$$\frac{\Delta}{P_e-\Delta}=\frac{\mu_{12}}{\lambda}=\frac{\frac{A_P}{a_P}-1}{1+A_P\left(\frac{q_I}{q_P}-1\right)}. \tag{8}$$

The last equation permits the calculation of (μ_{12}/λ) from the experimental data. As shown in a previous paper (Weber, 1954)

$$\frac{\mu_{12}}{\lambda}=\frac{4\pi}{15\times10^3}cN\frac{R^6}{(2a)^3}, \tag{9}$$

where c refers to the concentration of the acceptor molecules (indole, in our case). If the concentration of phenol is kept constant and that of indole is varied, a plot of $\Delta/P_e-\Delta$ against c should give a straight line from which the value of R may be calculated.

METHODS

The polarization of the fluorescence was measured with the arrangement shown in Fig. 1*a*. Light from a compact source Xe (375 w, xenon arc) was focused on the entrance slit of the grating monochromator G. The light emerging from the exit slit X is rendered parallel by the quartz lens L and falls on the solution contained in the quartz cell Q. The fluorescence emerging at right angles to the direction of excitation passes through the Glan–Thompson prism P and reaches the quartz-window photomultiplier (E.M.I. 6255). The photocurrents are measured directly by a Scalamp galvanometer with the polarizing prism in two positions. In one the polarized component normal to the directions of excitation and observation ($I_\parallel$) is measured. In the other polarizer position the component polarized in the direction of the exciting light is measured ($I_\perp$). The polarization p is given by

$$p\equiv\frac{I_\parallel-I_\perp}{I_\parallel+I_\perp}. \tag{10}$$

To reduce the Brownian molecular rotations so that the relaxation time of the rotation becomes very large compared with the lifetime of the excited state, a solvent with a viscosity of a few poises is required. Glycerol, which is currently used for this purpose, has poor transparency in

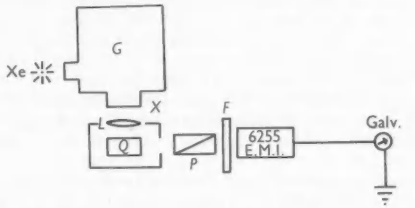


Fig. 1*a*. Apparatus for the determination of the polarization spectra. Xe, 375 w xenon arc; G, grating monochromator (dispersion 3·3 mμ/mm. at the slit); L, quartz lens, f 3 cm.; Q, spectrasil cuvette or block; P, Glan–Thompson prism; F, fluorescence filter; E.M.I. 6255, 14-stage, quartz-window photomultiplier; Galv., Scalamp galvanometer.

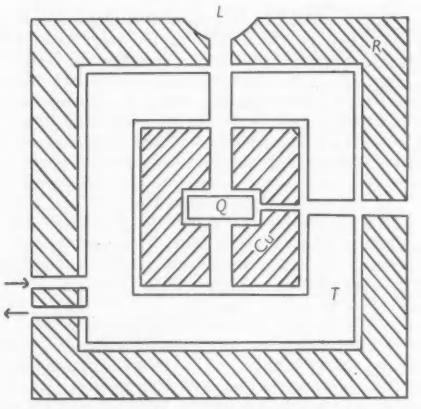


Fig. 1*b*. Section of cell container. R, Expanded rubber layer; T, Brass tank with inlet and outlet for circulation; Cu, copper block to position cuvette on block and serve as heat reservoir; Q, spectrasil cuvette or block.

the ultraviolet region, due to impurities which are often strongly fluorescent. Attempts at purification of the glycerol by chromatography or distillation were not successful. Propylene glycol (British Drug Houses Ltd., AnalaR) proved both transparent and free from fluorescent impurities. Its viscosity at room temperature is only about 0·6 poise, but at $-70°$ it forms a stiff glass with a viscosity of many poises (Williams, 1957), in which observations can yield the actual pseudo-principal polarization. To cool the cell or quartz block to this temperature, it was surrounded by a solid copper block which in turn was placed inside a brass jacket through which ethanol cooled by solid CO_2 was rapidly circulated from a Dewar vessel by means of a pump. The jacket was surrounded by a 1 in. thick insulating layer of expanded rubber and fibre glass (Fig. 1*b*). The metal block, the brass jacket and the insulating coat had channels at the front and side through which the exciting and fluorescent light passed. The lens L was in direct contact with the front opening and the polarizer rim with the side opening, of these channels. In this way circulation of air in contact with the cell was prevented and condensation of moisture on the optical surfaces avoided. A temperature of $-69°$ to $-70°$ could be constantly maintained in the liquid returning from the jacket.

The Glan–Thompson prism used was opaque to wavelengths shorter than 280 mμ. Its transmission above 300 mμ was very good, allowing the fluorescence of phenol, cresol and tyrosine ($\lambda_{max.}$ 303 mμ) to be satisfactorily measured.

The light emerging from the grating monochromator was found to be partially polarized to a small extent. Calling Ω the depolarization ratio of the exciting light, that is the ratio of its perpendicular to its parallel component, it can be found from the effect of each component independently that the polarization $\bar{p}$ observed is related to p_n, the polarization observable on excitation by natural light, by the equation

$$\bar{p} = \frac{2p_n}{1+p_n+\Omega(1-p_n)}. \qquad (11)$$

The light emerging from the monochromator was polarized to the extent of less than 10%, the magnitude of this polarization changing along the spectrum. However, as equation (11) shows, partial polarization of 10% makes $\bar{p}$ differ from p_n by about 5%. Since the precision of the polarization measurements was not much better than this no correction for polarization of the exciting light was applied. The smallest exciting band width consistent with reasonable accuracy was employed. Over the region 230–260 mμ the band widths employed were 3·0–1·5 mμ and 1·5–0·8 mμ in the region 270–310 mμ. A filter (F, Fig. 1) was interposed as usual between fluorescent source and detector to eliminate scattered and parasitic exciting light. With cresol, phenol and tyrosine the filter was a 4 mm. layer of Perspex plastic, which eliminated light of wavelengths shorter than 280 mμ. With indole, tryptophan and derivatives a 3 mm. layer of 0·02 M-$CuSO_4$ in 0·1 M-2-amino-2-hydroxymethylpropane-1:3-diol buffer (tris), pH 8·3, in a flat Perspex container was used. This was found to eliminate light of wavelength shorter than 315 mμ very satisfactorily.

In studying the fluorescence of concentrated solutions it is necessary to eliminate the depolarization that results from absorption of the fluorescence and its secondary emission. This radiative transfer of the excited state, the importance of which has been overlooked in the past, can lead to serious over-estimation of the non-radiative transfer. Birks & Little (1953), Bailey & Rollefson (1953) and Schmillen (1953) have shown directly the lengthening of the lifetime of the excitation due to radiative transfers in thick layers of fluorescein in solution and in solutions and crystals of anthracene. Schmillen has shown that this lengthening is absent in thin layers in which reabsorption of the fluorescence is negligible. Weber (1956) has shown that the depolarization of the fluorescence observed in thick layers of relatively dilute solutions disappears when thin layers of the same concentration are observed. Thus the observation of the polarization in very thin layers of solution is indispensable if the radiative transfer is to be ruled out. If the fraction of the total fluorescence absorbed inside the layer is β and the quantum yield of the fluorescence is q, a fraction $(1-\beta)$ of the radiation excited by the external source (primary fluorescence) will reach the detector and fraction β will be absorbed and will excite secondary fluorescence that reaches the detector with intensity $\beta q(1-\beta)$. In the same way the tertiary fluorescence equals $\beta^2q^2(1-\beta)$, etc. The total fluorescence due to reabsorption inside the layer finally reaching the detector equals:

$$(1-\beta)\,[\beta q+(\beta q)^2+(\beta q)^3+\ldots] = (1-\beta)\left(\frac{\beta q}{1-\beta q}\right). \qquad (12)$$

The ratio of internally excited fluorescence to primary fluorescence is simply $\beta q/(1-\beta q)$. In the preceding calculation no account has been taken of the geometry of the system. This can be disregarded only when the volumes illuminated and observed are the same and no illuminated volume is excluded from observation. Only in this case do the primary and higher orders of fluorescence have equal chances of reaching the detector. This situation can be realized in practice by using a thin layer of solution. The exciting beam is limited so that the whole illuminated zone falls within the aperture of the photomultiplier which is in turn restricted so as to include the illuminated zone alone.

If equation (12) is used to calculate the proportion of internally to externally excited fluorescence in a 30 μ layer of phenol solutions with q 0·2 and where the molar absorption coefficient for its own fluorescence is of the order of 100, it is found that in a 0·5 M-solution the internally excited fluorescence amounts to 5% of the total. At lower concentrations, and at all the concentrations of indole employed, the contribution of the internally excited fluorescence was negligible. The actual experimental arrangement consisted of two prisms of spectrasil quartz with optically flat faces. The solution was applied to the hypotenuse faces, which were then pressed firmly together. In this way a layer of very reproducible thickness of 30 μ was obtained. All the experiments on concentrated solutions, both of fluorescence polarization and fluorescence spectra, were carried out with these thin layers.

Determination of the contribution of phenol and indole to the fluorescence spectra of mixtures

The fluorescence spectra were determined as previously described (Teale & Weber, 1957). The fluorescent source was a layer of solution of 30 μ thickness excited by the light from a 375 w xenon arc, filtered through a Chance OX7 filter. The band-width employed was 1 mμ.

To determine the extent of the energy transfer from phenol to indole in the solutions, it is required to determine the ratio P/I of the photons emitted as phenol and indole fluorescence, and compare this with the ratio $(P/I)_e$ that would be expected in the absence of transfer. If n_P and n_I are the numbers of photons absorbed by the phenol and indole from the exciting beam and q_P and q_I the corresponding quantum yields of the fluorescence,

$$(P/I)_e = n_P q_P / n_I q_I .$$

Moreover,

$$\frac{n_P}{n_I} = \frac{\int_{\lambda a}^{\lambda b} I(\lambda)\,[1-10^{-(E_I+E_P)}]\,\dfrac{E_P}{E_I+E_P}\,d\lambda}{\int_{\lambda a}^{\lambda b} I(\lambda)\,[1-10^{-(E_I+E_P)}]\,\dfrac{E_I}{E_I+E_P}\,d\lambda}. \quad (13)$$

Of the three factors appearing in the integrals, $I(\lambda)$ is proportional to the photon output of the source after passing through the appropriate excitation filter, in our case a 3 mm. thickness of Chances's OX7 filter; $1-10^{-(E_P+E_I)}$ is the fraction of this radiation of a given wavelength absorbed in the layer, and $E_I/(E_I+E_P)$ and $E_P/(E_I+E_P)$ are respectively the fractions of the total absorption due to indole and phenol respectively. E_I and E_P are the separate extinctions of the indole and phenol layers. The additivity of their contribution is shown clearly in Fig. 2. In practice $I(\lambda)$ is determined as already described (Teale & Weber, 1957) and the E values by spectrophotometry of the thin layers. The integrals were computed graphically, the practical limits of integration being 240 and 305 mμ. The values of q_P 0·20 and q_I 0·42 are already in the literature (Weber & Teale, 1957). The ratio P/I is determined as follows: let $F_1 = P_1 + I_1$ represent the total fluorescence emitted at wavelength λ_1, at which wavelength we expect a dominant contribution from phenol. Similarly, $F_2 = P_2 + I_2$ is the total fluorescence at wavelength λ_2 at which the contribution from indole is dominant. The coefficients α_{12} and α_{21} obtained from observations of pure indole and phenol solutions are given by:

$$I_1 = \alpha_{21} I_2,$$

$$P_2 = \alpha_{12} P_1,$$

so that

$$\frac{P_1}{I_2} = \frac{F_1 - \alpha_{21} F_2}{F_2 - \alpha_{12} F_1}. \quad (14)$$

To relate the fluorescence emission at the given wavelengths to the total fluorescent emission P and I, a correction for the unequal spectral distribution of the separate fluorescences is required. Let the photon-fluorescence emission from each substance be described by two half-Gaussian distributions commencing at the wavelength of maximum emission, with intensity $P_{\max.}$ or $I_{\max.}$ and having standard deviations σ_+ towards the long-wave side and σ_- towards the short-wave side. The total emissions are proportional to $P_{\max.}(\sigma_+ + \sigma_-)_P$ and $I_{\max.}(\sigma_+ + \sigma_-)_I$. Therefore:

$$\frac{P}{I} = \frac{P_1 \alpha_{1m} (\sigma_+ + \sigma_-)_P}{I_2 \alpha_{2m} (\sigma_+ + \sigma_-)_I}. \quad (15)$$

In the last equation the coefficients α_{1m} and α_{2m} relate the fluorescence emitted by pure indole and phenol solutions respectively at the wavelength of maximum emission $P_{\max.}$ and $I_{\max.}$ to the emissions at wavelengths λ_1 and λ_2, that is to P_1 and I_2. Finally, if the ratio P/I is to represent the ratio of the numbers of photons leaving the fluorescent solution it has to be corrected by multiplication by factors representing the ratio of the attenuations of the two fluorescences by the grating and the difference in response of the photomultiplier. Because of the vicinity of the spectra these factors are not very different from 1. They have been determined as described by Teale & Weber (1957).

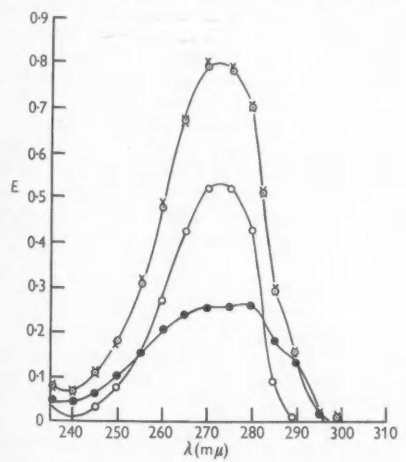


Fig. 2. Absorption spectra of phenol and indole in propylene glycol in 30 μ layers. ●, 0·02 M-Indole; ○, 0·2 M-phenol; ⊙, 0·02 M-indole + 0·2 M-phenol (calc.); ×, 0·02 M-indole + 0·2 M-phenol (observed).

RESULTS

Polarization spectrum of phenol, cresol and tyrosine

As shown in Fig. 3, the spectra are very similar. They consist of two regions, one of positive polarization that reaches a maximum value of 0·21–0·22 in

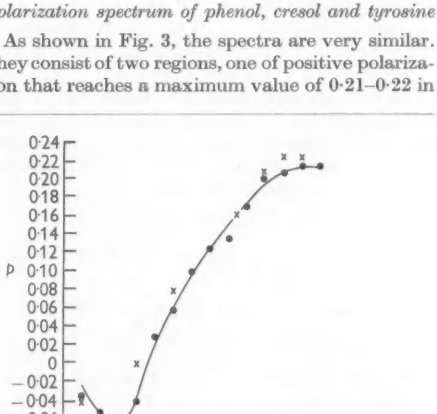
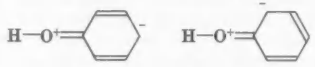


Fig. 3. Polarization (P) spectra of cresol (×) and tyrosine (●), 0·5 mM in propylene glycol, at −70°.

the 275–290 mμ region. The negative polarization band has a minimum of $-0{\cdot}065 \pm 0{\cdot}004$ at 235 mμ. The increase in the absolute polarization observed at wavelengths shorter than 235 mμ is due to a contribution to the absorption from a band having its maximum in the vacuum ultraviolet region. The transition with maximum at 275 mμ in phenol and phenolic derivatives may be expected to have a large contribution by the quinonoid forms to the



excited state, as evidenced by the large increase in absorption and shift to longer wavelengths observed in the phenolate ion. It may be expected that if in this type of transition an electron from the oxygen lone pair is promoted to an excited orbital in the ring ($n \rightarrow \pi^*$ transition) the direction of the transition moment will be normal to the plane of the ring, and therefore at right angles to the direction of the transition moment of the 235 mμ absorption, which must be expected to be in the ring plane as all $\pi \rightarrow \pi^*$ transitions are. On the other hand, both transitions may be normal to each other while still contained in the plane of the ring. The distinction between the two cases may be made in practice by a study of the dichroism of crystals if the absolute orientation of the benzene ring is known, and should furnish a criterion of the $n \rightarrow \pi^*$ character (as opposed to $\pi \rightarrow \pi^*$ character) of the 275 mμ absorption. An equation due to Jablonski (1935) permits the calculation of $\cos^2\beta_i$, where β_i is the angle between the ith oscillator of absorption and the emission oscillator:

$$\cos^2\beta_i = \frac{7p_i + 1}{3 + p_i}, \qquad (16)$$

where p_i is the polarization observed by excitation with natural light. Although the angle δ between the absorption oscillators cannot be calculated directly, if they and the emission oscillator are all contained in the same plane and

$$\delta = \tfrac{1}{2}\pi, \quad \cos^2\beta_1 + \cos^2\beta_2 = 1.$$

From the values of p observed for the two transitions and equation (16),

$$\cos^2\beta_1 = 0{\cdot}185; \quad \cos^2\beta_2 = 0{\cdot}78;$$

$$\cos^2\beta_1 + \cos^2\beta_2 = 0{\cdot}965.$$

Thus the directions of the transition moments associated with the 235 and 275 mμ absorption bands of phenol or tyrosine appear to be at right angles to each other. Equation (16) would be expected to be approximately correct even if the direction of the transition moment in emission is not sharp but, as suggested by Jablonski (1950), comprises a set of directions determined by the torsional vibrations of the molecule in the excited state.

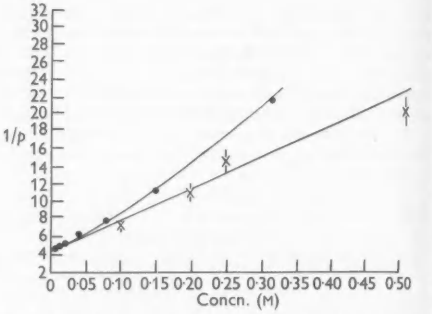


Fig. 4. Concentration depolarization in phenol solutions in propylene glycol at −70°: ×, 30 μ layers; ●, 5 mm. layers.

Concentration depolarization in phenol solutions

The data are shown in Fig. 4, where the reciprocal of the polarization is plotted against the phenol concentrations. The observations refer to thin layers of 30 μ thickness and thick layers of 5 mm. thickness. The linear law is approximately followed by the thin-layer fluorescence. In the thick layers the increase in depolarization at high concentrations is due to radiative transitions, but at the lower concentrations, where radiative transfer may be expected to be absent, the data agree well with those of the thin layers. From equation (6), $R = 17$ Å. On the other hand, from Förster's theory, with $n = 1{\cdot}5$; $\tau = 8$ μmsec.; $\frac{1}{2}(\nu_a + \nu_e) = 34\,700$ cm.$^{-1}$; $\nu_a - \nu_e = 3400$ cm.$^{-1}$; $\sigma_a \simeq \sigma_e = 1500$ cm.$^{-1}$; $E_1 = E_2 = 1600$ cm.^{2}/m-mole; equation (4) gives $J_v = 3{\cdot}2 \times 10^8$ cm.^{3}/m-mole^2, and equation (5) gives $R_c = 11$ Å. The value predicted by the theory is apparently considerably smaller than the experimental value, but this may be due, at least partly, to the fact that equation (5) takes into account only the dipole–dipole interaction between the molecules and neglects quadrupole and higher terms.

Polarization spectrum of indole, N-methylindole and tryptophan

These spectra are shown in Fig. 5. The three substances have similar spectra, showing two minima, one at 232 ± 2 mμ the other at 290 mμ, and two maxima at 265–270 and 300–305 mμ respectively. The minimum at 232 mμ coincides with the maximum of one of the absorption bands. The maximum at 270 mμ does not correspond exactly with the maximum of the longest wavelength-absorption band and the minimum at 290 mμ does correspond accurately with the

shoulder observed in the absorption spectrum. The maximum at 310 mμ and a shallow minimum found at longer wavelengths do not have any corresponding features in the absorption spectrum, which at these wavelengths consists only of a weak tail. All the details of the polarization spectrum, up to and including the 310 mμ maximum, can be explained on the likely assumption that the longest wavelength-absorption band of tryptophan consists of two independent electronic transitions (G–S_1 and G–S_2), of which one (G–S_2) is responsible for the shoulder observed in absorption at 290 mμ. Fig. 6 indicates a possible disposition of the potential-energy surfaces involved in the transitions: r is a co-ordinate or average of co-ordinates which best describes the nuclear-configuration changes taking place in the excited state; V is the potential energy. According to the Franck–Condon principle the absorption takes place from the centre of the zero level of the potential-energy surface of the ground state G, reaching the singlet states S_1 and S_2 placed vertically upwards. At wavelengths at which $V > V_2$, excitation to both S_1 and S_2, but principally to S_1, takes place. This corresponds to the region of the absorption spectrum where the polarization increases monotonically. For energies between V_2 and V_1 the excitation to S_2 predominates and shows itself in a decrease in the polarization since the transition moment of the G–S_2 transition determines a low, perhaps negative, polarization when excited alone. At energies $V < V_1$ the Franck–Condon principle determines that the absorption to S_2 must disappear completely, but weak absorption to S_1 may still take place. Since S_1 is excited alone the polarization increases rapidly, well above the 270 mμ maximum at which both S_1 and S_2 are excited, whereas the absorption drops to a weak tail. The maximum at 305 mμ is in all probability due to the excitation of S_1 alone, but it is not possible to decide whether S_2 has an actual negative polarization since it is not excited alone at any wavelengths. The transition in fluorescence is the converse of the G–S_1 transition and takes place therefore from the minimum of the S_1 surface. In agreement with the above scheme the fluorescence bands of indole and tryptophan are found to be unresolved and symmetric and to show no detail on the short-wave side corresponding to the fine-structure band or shoulder in absorption at 290 mμ. The separation of the absorption and fluorescence bands is considerable (3400 cm.$^{-1}$ in indole in propylene glycol, 5000 cm.$^{-1}$ in tryptophan in water) and the changes in the position of the absorption and fluorescence bands with the dielectric properties of the medium (Lippert, 1957) indicates the loss of energy of the excited state by dipolar interaction with the solvent molecules during the lifetime of the excited state. The fluorescence transition may be described as taking place between S_1' and G. The energy difference $S_1 - S_1'$ (Fig. 6) is the energy loss by dipolar interaction with the solvent molecules. The transitions G–S_1 and G–S_2 may be

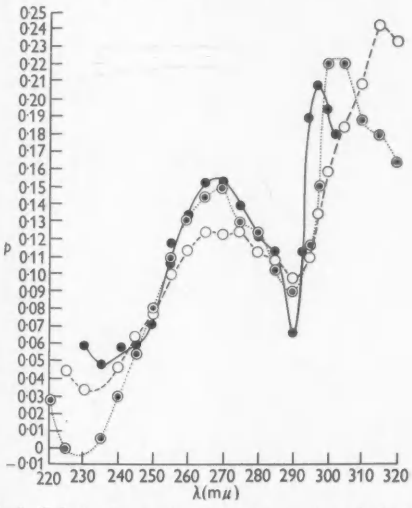


Fig. 5. Polarization spectra of indole (●), N-methylindole (○) and tryptophan (⊙) in propylene glycol at −70°. Concentration 0·2 mM.

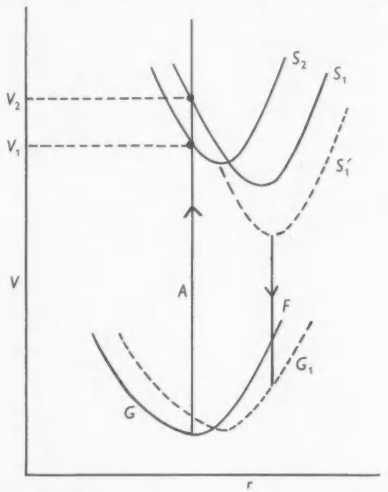


Fig. 6. Potential-energy surfaces for the ground state G, the two singlet-excited states S_1 and S_2 and the fluorescent state S_1', of indole.

provisionally identified with the L_a and L_b transitions in the theory of the absorption spectrum of aromatic hydrocarbons due to Platt (1949). The L_a transition is responsible for the longest wavelength-absorption band. In indole solutions in hexane our G–S_1 transition shows five distinct peaks, in correspondence with similar five peaks of vibrational structure present in the L_a transitions of benzene and anthracene. The overlap of the L_a and L_b transitions has also been suggested by Weller (1957) to explain the absorption spectrum of acridine.

Polarization spectrum of N-glycyltryptophan

The spectra, shown in Fig. 7, are like those of indole and tryptophan with one important difference: the appearance of a new polarization band, with a band width of a few millimicrons, preceding the 295 mμ minimum. This band is difficult to reproduce in practice because of the inaccuracy in repeating the setting of the exciting wavelengths. As shown in the following paper this band appears also in the proteins, and since it is not present in the simple indole derivatives it is probably connected with the existence of the peptide bond. The characteristics of the band appear the same in 50% (v/v) propylene glycol–water and in 50% propylene glycol–water containing 6 M-urea. This result appears to indicate that a close external interaction of the indole ring with the peptide bond is not required for its appearance.

Concentration depolarization in indole solutions

If equations (4) and (5) are used to calculate the value of R_c for propylene glycol solutions of indole, for which

$$\sigma_a \simeq \sigma_e = 3000 \text{ cm.}^{-1},\ E_1 = E_2 = 5600 \text{ cm.}^2/\text{m-mole},\ \tfrac{1}{2}(\nu_a + \nu_e) = 33\,200 \text{ cm.}^{-1}$$

and $\tau \simeq 10$ μsec., it is found that $J_v = 1{\cdot}8 \times 10^{10}$ cm^3/m-mole^2, $R_c = 23$ Å. The polarization spectra of solutions of several concentrations (0·05–0·4 M) are recorded in Fig. 8 to show an important property: the polarization decreases rapidly with increasing concentration when the fluorescence is excited with light of wavelength shorter than 300 mμ but appears hardly changed if the 305–315 mμ range is used. Thus a 0·4 M-solution excited with 305 mμ shows over 70% of the polarization of the dilute solution, but excited with 270 mμ the polarization is less than 15% of that of the dilute solution. When $1/p$ is plotted against concentration the value of R calculated from the slope equals 16 Å, falling well short of the expected 23 Å. The experimental facts appear to indicate that excitation of the S_2 band is required for the transfer to take place and excitation of S_1 alone is ineffective. It may be argued that this is a case in which a long-wave limit is

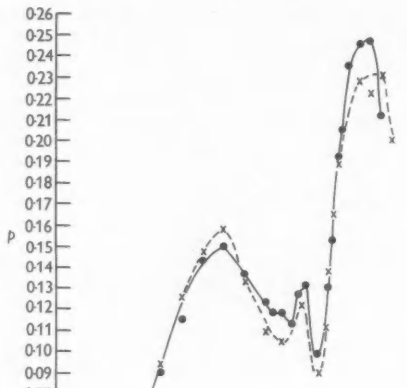


Fig. 7. Polarization spectra of 0·2 mM-*N*-glycyltryptophan in propylene glycol at −70°. ●, In 50% propylene glycol–water; ×, in 50% propylene glycol–water containing 6 M-urea.

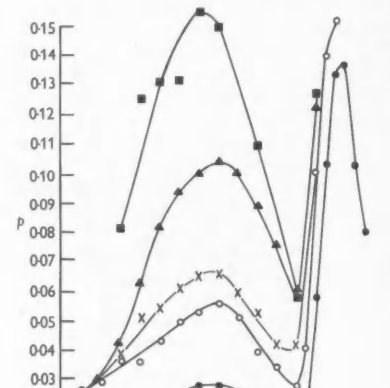


Fig. 8. Polarization spectra of 30 μ layers of 0·1–0·4 M-indole in propylene glycol at −70°. ■, 0·01 M; ▲, 0·05 M; ×, 0·1 M; ○, 0·2 M; ●, 0·4 M.

found for the transfer because only at wavelengths well above the fluorescent level it is possible to match the given and accepted energies (Moodie & Reid, 1952). In the present case, however, the long-wave limit of the transfer appears sharp and coincident with the limit of an electronic transition.

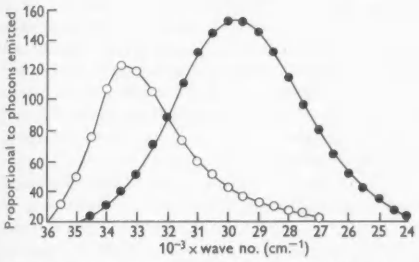


Fig. 9. Fluorescence spectra of phenol and indole in propylene glycol at room temperature, corrected to equal number of photons absorbed. Areas are thus proportional to the quantum yields of fluorescence. ○, Phenol; ●, indole.

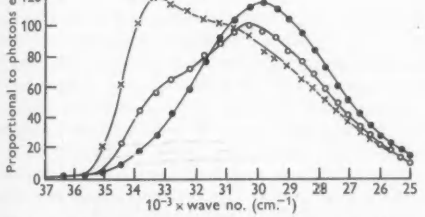


Fig. 10. Fluorescent spectra of 30 μ layers of solutions of phenol and indole in propylene glycol at room temperature. ×, 0·01 M-Indole in M-phenol; ○, 0·03 M-indole in M-phenol; ●, 0·1 M-indole in M-phenol.

Table 1. *Fluorescence emitted by indole and phenol in mixtures of fixed phenol concentration* (1 M) *and variable indole concentration* (C)

$a_p = P/(P+I)$ and $A_P = P_e/(P_e+I_e)$ are respectively the observed and expected fractions of the total fluorescence emitted as phenol fluorescence. $\Delta/(P_e-\Delta)$ has been calculated by equation 8 with $q_I/q_P = 2{\cdot}1$.

C (mM)	A_P	a_p	$\Delta/(P_e-\Delta)$
5	0·945	0·78	0·10
10	0·89	0·59	0·26
20	0·70	0·42	0·38
30	0·57	0·29	0·59
50	0·43	0·17	1·04
100	0·26	0·06	2·59

Moreover, as described below, the transfer of the excitation from phenol to indole results in the appearance of a weak negative polarization in the indole fluorescence. This is only possible if the indole transition involved in the transfer would yield a negative polarization when directly excited. From the data for the polarization spectrum of indole it appears that only the G–S_2 transition can fulfil this condition. If the S_2 transition must be excited for transfer to occur it follows that use of the value for the total lifetime of the excited state in the calculation of R_c must lead to its overestimation. If the lifetime of the excitation in the S_2 band is 1 μsec. then $R_c = 15$ Å; if it is 0·1 μsec. then $R_c = 11$ Å. The latter value for the lifetime is probably an upper limit for the vibrational deactivation that precedes emission (e.g. Weber & Teale, 1958), so that as with phenol, and probably because of similar reasons, R considerably exceeds R_c.

Transfer of the excited state from phenol to indole

This was determined by a study of the fluorescence spectra of molar solutions of phenol in propylene glycol containing increasing concentrations of indole, from 5 mM to 0·1 M. The fluorescence spectra determined are shown in Figs. 9 and 10. The possibility of a ground-state complex of indole and phenol which would invalidate the calculation of R_c based on the random distribution of the indole, with respect to the excited phenol molecules, appears to be excluded by a study of the absorption spectrum of the mixtures, which appeared to be the exact sum of the phenol and indole contributions (Fig. 2). Table 1 gives the experimental values of $A_p = P_e/(P_e+I_e)$ and $a_p = P/(P+I)$. The ratios P_e/I_e were calculated by the use of equation (12). P/I is calculated as already discussed from the total fluorescent emission at the wavelengths, λ_1 300 mμ and λ_2 340 mμ. The data for the fluorescence spectrum of pure indole and phenol in propylene glycol at room temperature (Figs. 8 and 9) required to calculate P and I by the use of equations 14 and 15 are:

	Phenol fluorescence	Indole fluorescence
$\lambda_{max.}$	303 mμ	335 mμ
σ^+	1750 $cm.^{-1}$	3100 $cm.^{-1}$
σ^-	1300 $cm.^{-1}$	2800 $cm.^{-1}$
	$\alpha_{12} = 0{\cdot}155$	$\alpha_{21} = 0{\cdot}156$
	$\alpha_{1m} \simeq 1$	$\alpha_{2m} \simeq 1$

The corrections for grating transmission and detector response are:

$$\frac{\text{grating transmission for phenol fluorescence}}{\text{grating transmission for indole fluorescence}} = 0{\cdot}94$$

$$\frac{\text{detector response for phenol fluorescence}}{\text{detector response for indole fluorescence}} = 1{\cdot}25.$$

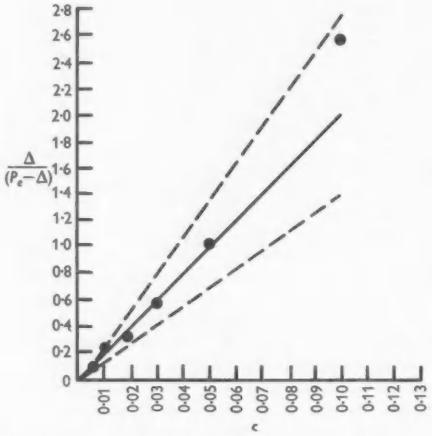


Fig. 11. Plot of $\Delta/(P_e-\Delta)$ against indole concentration (c) (data of Table 1). The broken lines correspond to slopes giving respectively R 17·5 Å and 19·5 Å. The continuous line has slope corresponding to 18·5 Å.

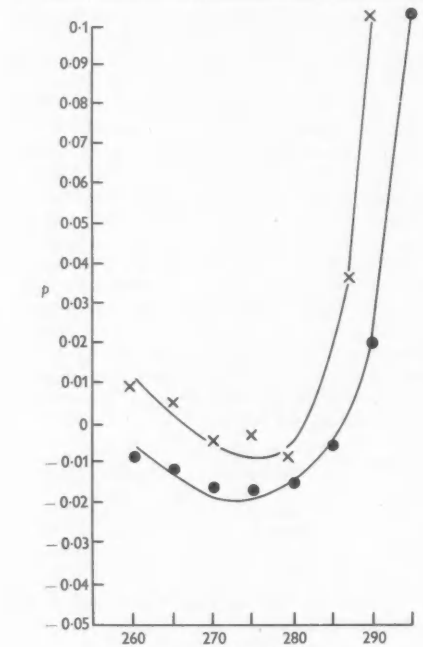


Fig. 12. Polarization spectra of mixtures of phenol and indole in 30 μ layers to show the appearance of negative polarizations. ●, 0·01 M-Indole in M-phenol; ×, 0·05 M-indole in M-phenol.

So that finally $P/I = 0{\cdot}807$ (P_1/I_2). Fig. 11 shows a plot of $\Delta/(P_e-\Delta)$ [calculated by means of equation (8)] against c, the indole concentration. The expected linear relation is followed at least up to a concentration of indole of 0·05 M. In comparing the values of R and R_e the probable error on the determined values of these quantities must be considered. If the standard deviation of the straight-line plots from which R is calculated equals $\delta S/S$, the error in R is given by equation (9), as $\delta R/R \simeq \frac{1}{6}\delta S/S$. Similar remarks apply to the calculation of R_e from J_ν: $\delta R_e/R_e \simeq \frac{1}{6}\delta J_\nu/J_\nu$. Granting the correctness of the assumptions involved in the derivations, the R and R_e values should be accurate to within 1 Å.

With the values already quoted for the spectroscopic constants of the indole absorption and phenol fluorescence, and by the use of equations (4) and (5), we obtain $J_\nu = 1{\cdot}04 \times 10^{10}$ cm.^{3}/m-mole^2, R_e 19 Å, and the slope of the straight line in Fig. 11 gives $R = 18{\cdot}5$ Å. The rather accidental character of the agreement between the two values must be suspected in view of the disagreement found in other cases (Weber, 1954; Weber & Teale, 1959).

Polarization of the fluorescence of phenol–indole mixtures

The polarization spectrum of the fluorescence of some of the solutions was measured with a copper-tris liquid filter to attenuate preferentially the phenol fluorescence. This filter transmitted 10 % of the phenol fluorescence and 25 % of the indole fluorescence. For this reason the fluorescence observed on excitation by wavelengths shorter than 300 mμ can be given only qualitative significance. The results are given in Fig. 12, which shows a small but doubtless negative polarization, in the region and at the concentrations in which indirect excitation through the phenol is more prominent. Such negative polarization can only be expected if the oscillator active in the transfer on the indole side would give itself a negative polarization when directly excited. Although negative polarization is not observed at any wavelength in the spectrum of indole (Fig. 5), this is probably due to the fact that the S_2 transition cannot be excited alone. The fact that the S_2 transition appears as the active one in the phenol–indole transfer agrees with the sharp long-wave limit observed already in the indole–indole transfer.

SUMMARY

1. The polarization of the fluorescence as a function of the exciting wavelength (polarization spectrum) has been determined from 230 mμ to the long-wave absorption limit for solutions of tryptophan, tyrosine and some related substances, in

propylene glycol solution at $-70°$, excited with unpolarized light.

2. Phenol, cresol and tyrosine show a simple spectrum, consisting of a negative polarization region with a minimum value of $-0{\cdot}065$ at 235 mμ, and a positive region with a maximum of 0·22 at 275 mμ and longer wavelengths.

3. Indole, *N*-methylindole and tryptophan show no negative polarization at any wavelengths. In the region of the longest wavelength-absorption band two maxima are present at 270 and 305 mμ and a minimum at 295 mμ. It is shown that at least two electronic transitions are required to describe these effects, one of these transitions corresponding to the spike or shoulder observed in absorption at 295 mμ.

4. *N*-Glycyltryptophan has a spectrum similar to the other indole derivatives but for the existence of an additional sharp polarization band with maximum at 290 mμ, which appears connected with the presence of a peptide bond.

5. Concentration-depolarization measurements show that the distance R at which the probability of transfer of the excitation equals the probability of emission is 17 Å for phenol molecules.

6. Concentration-depolarization measurements on indole solutions show that $R = 17$ Å if the fluorescence is excited with light of wavelength shorter than 295 mμ, but that excitation with longer wavelengths is not followed by transfer with very much lower efficiency.

7. Measurements of the fluorescence spectrum in mixtures of phenol (1 M) and indole (0·01–0·2 M) show that phenol can transfer its energy to indole with R 18·5 Å.

REFERENCES

Bailey, E. A. & Rollefson, G. K. (1953). *J. chem. Phys.* **21**, 1315.
Birks, J. B. & Little, W. A. (1953). *Proc. phys. Soc. Lond.*, A **66**, 921.
Förster, T. (1947). *Ann. Phys., Lpz.*, **2**, 55.
Jablonski, A. (1935). *Z. Phys.* **96**, 236.
Jablonski, A. (1950). *Acta phys. polon.* **10**, 33, 193.
Lippert, E. (1957). *Z. Elektrochem.* **61**, 962.
Moodie, M. M. & Reid, C. (1952). *J. chem. Phys.* **20**, 1510.
Oppenheimer, J. R. & Arnold, W. (1950). *J. gen. Physiol.* **33**, 423.
Perrin, F. (1926). *J. Phys. Radium*, **7**, 390.
Perrin, F. (1929). *Ann. Phys., Paris*, **12**, 169.
Perrin, F. (1932). *Ann. Phys., Paris*, **17**, 283.
Platt, J. R. (1949). *J. chem. Phys.* **17**, 484.
Schmillen, A. (1953). *Z. Phys.* **135**, 294.
Soleillet, P. (1929). *Ann. Phys., Paris*, **12**, 23.
Sveshnikoff, B. & Feofilov, P. P. (1940). *J. Phys., Moscow*, **3**, 493.
Teale, F. W. J. & Weber, G. (1957). *Biochem. J.* **65**, 476.
Weber, G. (1954). *Trans. Faraday Soc.* **50**, 552.
Weber, G. (1956). *J. opt. Soc. Amer.* **16**, 962.
Weber, G. & Teale, F. W. J. (1957). *Trans. Faraday Soc.* **53**, 646.
Weber, G. & Teale, F. W. J. (1958). *Trans. Faraday Soc.* **54**, 640.
Weber, G. & Teale, F. W. J. (1959). *Disc. Faraday Soc.* **27**, 134.
Weller, A. (1957). *Z. Elektrochem.* **61**, 956.
Williams, R. (1957). *J. chem. Phys.* **26**, 1187.

Biochem. J. (1960) **75**, 345

Fluorescence-Polarization Spectrum and Electronic-Energy Transfer in Proteins

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The analysis of the polarization spectrum of the fluorescence of proteins is necessarily more difficult than that of the aromatic amino acids and simple derivatives described in the previous paper (Weber, 1960). The main differences arise because of the various types of Brownian molecular rotations which are possible in the protein, and also the possibility of energy transfer among the aromatic residues. The characteristic distances of transfer R for phenol–phenol, indole–indole and phenol–indole transfer calculated from concentration-depolarization and fluorescence-spectral data (Weber, 1960) are of the order of the distance between these residues in the protein molecule. Finally, the possibility that interaction of the indole nucleus with the highly polarizable backbone of the protein may lead to changes in the relative positions of the overlapping transitions in tryptophan must be kept in mind.

EXPERIMENTAL

This paper describes the fluorescence-polarization spectrum of two classes of globular proteins.

Class A proteins containing phenylalanine and tyrosine but no tryptophan. These proteins (Teale & Weber, 1959;

F. W. J. Teale, in preparation) have a fluorescence spectrum characteristic of tyrosine with a maximum emission at 303 mμ. The quantum yield of the fluorescence is 0·03–0·05, that is one-fifth to one-seventh of that of free tyrosine (Teale & Weber, 1957).

Class B proteins, containing tryptophan as well as tyrosine and phenylalanine. These proteins show only tryptophan fluorescence with wide variations in quantum yield (0·04–0·3) and wavelength of maximum emission (320–350 mμ) (F. W. J. Teale, in preparation). No appreciable tyrosine fluorescence can be discerned in the fluorescence spectrum.

Materials. Bovine-serum albumin, bovine γ-globulin, bovine fibrinogen and lysozyme were Armour preparations. Ribonuclease, chymotrypsin, chymotrypsinogen and rabbit-muscle lactic dehydrogenase were Worthington Biochemical Corp. (N.J., U.S.A.) preparations. Ovalbumin was three-times crystallized, as described by Warner (1954). Insulin was a specimen from British Drug Houses Ltd. Human-serum albumin was a gift from Dr J. L. Oncley.

Methods. Polarizations were measured as described in the preceding paper. With protein solutions, scattering of parasitic light can falsify the results. This parasitic light is the light of wavelength longer than the cut-off of the filter used to separate the exciting from the fluorescent light. A first-order correction for the scattered parasitic light was determined by introducing into the path of the exciting beam a filter similar to that used to separate excitation from fluoresence. The light passing through both filters is to a first approximation the parasitic light that would be scattered in the absence of the excitation filter. With both filters in position the components $I'_\parallel$ and $I'_\perp$ of the scattered parasitic light were measured independently. They were subtracted respectively from $I_\parallel$ and $I_\perp$, the components of the fluorescence light as measured with the excitation filter removed. The polarization was calculated from the corrected components in the ordinary way Weber (1960). The intensity of parasitic light in our monochromator was practically independent of the exciting wavelength and depended exclusively on the slit width. This had to be increased as shorter wavelengths were used, particularly in the region 230–250 mμ, because of the falling output of the xenon arc in this region. The correction described was applied when $I'_\parallel \leqslant 0{\cdot}2 I_\parallel$. For larger values of the scattered light the correction is not considered reliable, and this condition has determined the short-wave limit of the polarization spectra here described.

RESULTS

Class A proteins

Insulin, ribonuclease and zein were studied in 90–95 % propylene glycol–water mixtures at −70°. The spectra, shown in Fig. 1, are qualitatively similar to those of phenol, cresol or tyrosine, but the absolute values of the polarization reached in both the negative and positive regions of the spectrum are smaller than in the simpler derivatives. It does not seem that these differences can be attributed to any influence other than the energy transfer among the tyrosine residues, since practically all rotational depolarization must be absent under the conditions described. The probability of transfer will be determined by the average distance between the tyrosine residues. In the absence of more detailed information, assuming for simplicity that these tyrosines are spaced in the same way as in a solution, the effective molar concentration c_e in the proteins equals $10^3\,(n/V)$ where n is the number of tyrosine residues per molecule and V the anhydrous molar volume of the protein. The probability of transfer will be also determined by the characteristic distance R for inter-tyrosine transfer already computed (Weber, 1960) and the lifetime τ of the tyrosine fluorescence in the protein, as compared with the lifetime τ_0 of the tyrosine in the medium in which R has been determined. From the theory of concentration depolarization already developed (Weber, 1954),

$$\frac{1}{p}+\frac{1}{3}=\left(\frac{1}{p_\infty}+\frac{1}{3}\right)(1+Ac\tau_0)\,, \qquad (1)$$

where p is the polarization observed at concentration c, τ_0 the lifetime of the excited state and A a constant proportional to R^6. p_∞ is the polarization in dilute solution in which no appreciable intermolecular transfer of the excitation takes place. If the assumption is made that the processes that decrease the quantum yield of the fluorescence are competitive with the emission, then $\tau/\tau_0 = q/q_0$, where q_0 and q are respectively the yields of the tyrosine fluorescence when free and in the protein. From a study of the concentration depolarization of phenol solutions (Weber, 1960) $A\tau_0 \simeq 20$, and

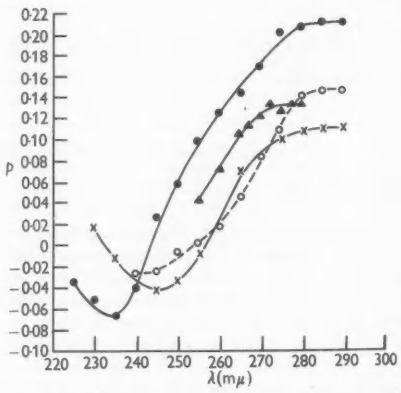

Fig. 1. Fluorescence-polarization spectra of insulin, zein and ribonuclease in propylene glycol at −70°. The spectrum of tyrosine is included for comparison. ●, Tyrosine; ▲, zein; ×, insulin; ○, ribonuclease.

therefore the polarization p expected in the protein is given by

$$\frac{1}{p}+\frac{1}{3}=\left(\frac{1}{p_\infty}+\frac{1}{3}\right)\left(1+20\,\frac{q}{q_0}\,c_e\right). \qquad (2)$$

p_∞ is the polarization of the fluorescence of tyrosine in a medium of infinite viscosity, which for excitation with 275 mμ wavelength equals 0·22. As a first approximation $\frac{1}{3}$ can be neglected by comparison with $1/p$ and, replacing c_e by its value $10^3\,(n/V)$,

$$p\simeq\frac{p_\infty}{1+\dfrac{2q}{q_0}\,\dfrac{n}{V}\,10^4}, \qquad (3)$$

with the values $2q/q_0\simeq\frac{1}{2}$ and $(n/V)\simeq 0{\cdot}5$ m-mole/ml.

$$p\simeq 0{\cdot}07.$$

The experimental values in the three proteins studied are all greater than this (0·11–0·14), although still considerably smaller than p_∞. A significant comparison of the observed with calculated values cannot be made since the quantum yield, orientation and relative position of the several tyrosine residues in the protein are not known.

Class B proteins

Qualitatively the polarization spectrum is that of tryptophan and glycyltryptophan. The spectra are shown in Figs. 2–10. The general features are given in paragraphs (1) to (6).

(1) The main polarization regions of tryptophan and other indole derivatives are present, with maxima at 270 mμ and 303–308 mμ and a minimum in the intervening region in every case.

(2) An intermediate band with a maximum at 290 mμ, having a shallow minimum on the short-wave side and a sharp minimum at 295 mμ and a band width of less than 10 mμ, similar to a band observed already in *N*-glycyltryptophan, is found in most proteins.

(3) Although the bands are qualitatively similar to those of the simpler indole derivatives there is an important quantitative difference, shown in Table 1. When the ratio of the polarizations observed on excitation by 270 mμ (p_{270}) and 305 mμ

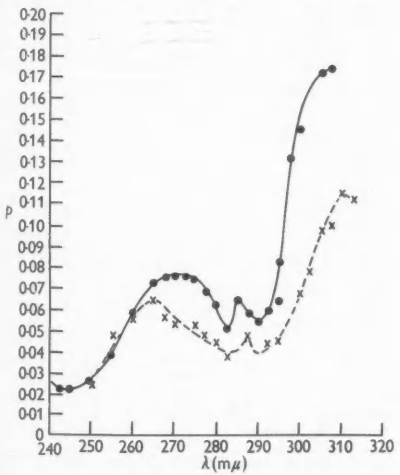

Fig. 2. Polarization spectra of human-serum albumin: ●, in buffer, pH 7·0; ×, in 8 M-urea. These symbols are used in the same way in Figs. 3–9.

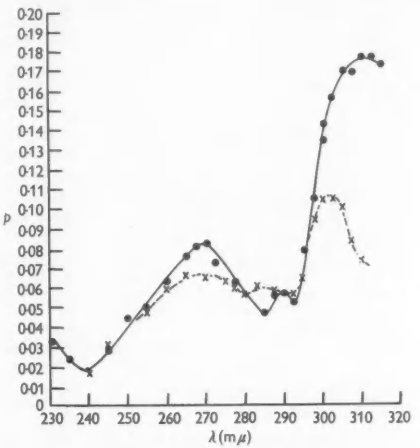

Fig. 3. Polarization spectra of bovine-serum albumin. See legend to Fig. 2.

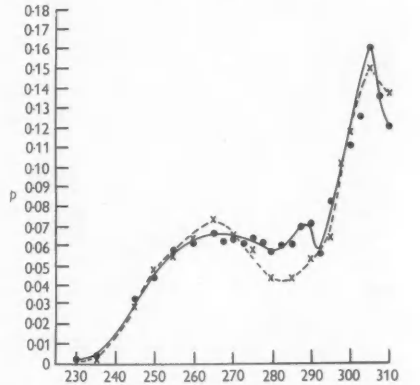

Fig. 4. Polarization spectra of pepsin. See legend to Fig. 2.

Table 1. *Class B proteins*

S.D. of the quoted values is $\Delta p \pm 0{\cdot}003$. 50% PG indicates measurements in 50% (v/v) propylene glycol–water mixture at $-70°$; PG indicates measurements in pure propylene glycol at $-70°$.

Protein	Solvent	p_{270}	p_{305}	$\frac{p_{305}}{p_{270}}$
Human-serum albumin	Buffer, pH 7·0	0·079	0·172	2·18
	8M-Urea	0·054	0·115	2·1
	50% PG	0·080	0·200	2·5
Bovine-serum albumin	Buffer, pH 7·0	0·084	0·172	2·1
	8M-Urea	0·084	0·117	1·6
	50% PG	0·080	0·200	2·5
Ovalbumin	Buffer, pH 7·0	0·083	0·140	1·7
	8M-Urea	0·054	0·080	1·5
Lysozyme	Buffer, pH 7·0	0·052	0·123	2·4
	8M-Urea	0·070	0·115	1·6
	50% PG	0·070	0·210	3
Bovine γ-globulin	Buffer, pH 7·0	0·075	0·149	2
	8M-Urea	0·070	0·105	1·55
	50% PG	0·081	0·178	2·2
Bovine fibrinogen	Buffer, pH 7·0	0·062	0·157	2·5
	8M-Urea	0·054	0·100	1·85
	50% PG	0·045	0·135	3
Chymotrypsinogen	Buffer, pH 7·0	0·057	0·154	2·7
	8M-Urea	0·064	0·092	1·4
	50% PG	0·048	0·154	3·2
Chymotrypsin	Buffer, pH 7·0	0·052	0·120	2·3
	8M-Urea	0·042	0·063	1·5
	50% PG	0·054	0·170	2·9
Pepsin	Buffer, pH 7·0	0·063	0·135	2·1
	8M-Urea	0·064	0·150	2·3
	50% PG	0·085	0·220	2·6
Rabbit-muscle lactic dehydrogenase	Buffer, pH 7·0	0·086	0·200	2·3
	8M-Urea	0·044	0·074	1·7
Tryptophan	PG	0·145	0·210	1·45
Indole	PG	0·155	0·205	1·33
N-Glycyltryptophan	PG	0·146	0·245	1·68

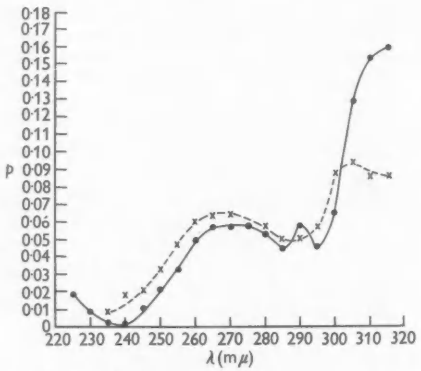

Fig. 5. Polarization spectra of chymotrypsinogen. See legend to Fig. 2.

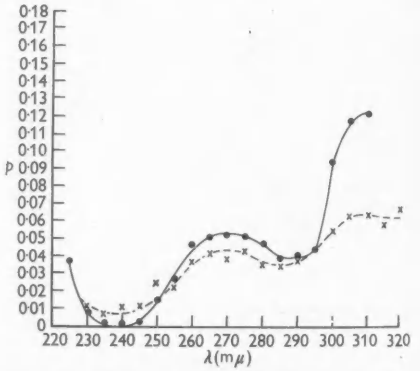

Fig. 6. Polarization spectra of chymotrypsin. See legend to Fig. 2.

(p_{305}) is recorded it is found that, for the simple molecules, $p_{305}/p_{270} = 1{\cdot}4$–$1{\cdot}7$, whereas for nine out of ten proteins $p_{305}/p_{270} > 2$. In ovalbumin the value is 1·7. This larger ratio in the proteins is due to a comparative decrease in the 270 mμ region. The values observed at 305 mμ approach more closely those encountered in the simple derivatives in a rigid medium (Table 1).

(4) A very small increase in polarization was found on dissolving the proteins in 60% sucrose. On the other hand, solutions of the proteins in 50% propylene glycol–water mixtures at $-70°$ showed considerable increases in the ratio p_{305}/p_{270}. In a series of eight proteins the ratio varied between 2·2 and 3·2 (Table 1 and Figs. 11–13). In all but one case (fibrinogen) the polarization at 305 mμ was increased, whereas p_{270} showed increases (lysozyme, pepsin), a decrease (fibrinogen) or remained the same (bovine and human albumin, γ-globulin, chymotrypsin).

(5) When the proteins were dissolved in 8 M-urea the two bands at 270 and 305 mμ persisted, but

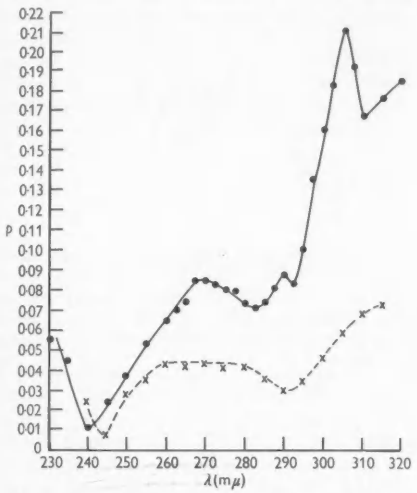

Fig. 7. Polarization spectra of rabbit-muscle lactic dehydrogenase. See legend to Fig. 2.

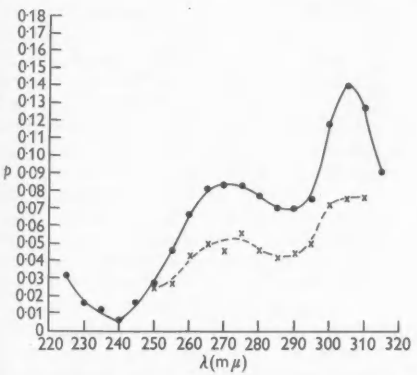

Fig. 8. Polarization spectrum of ovalbumin. See legend to Fig. 2.

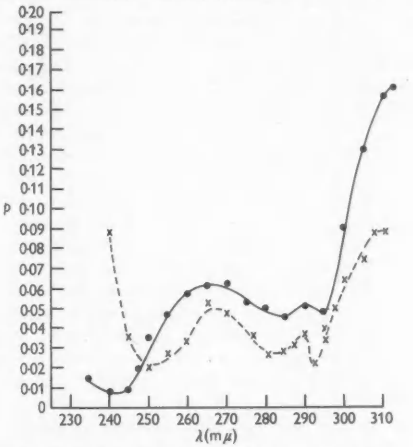

Fig. 9. Polarization spectrum of bovine fibrinogen. See legend to Fig. 2.

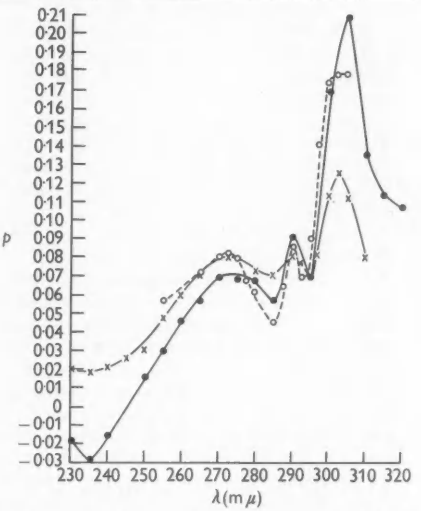

Fig. 10. Polarization spectra of lysozyme: ○, in buffer, pH 7·0; ×, in 8 M-urea; ●, in 50% propylene glycol–water at $-70°$.

they appeared usually much flatter than in the water solutions (Table 1). A decrease in the absolute values of both maxima was generally observed, but the fall in the 305 mμ maximum was much more pronounced, so that in all but one case (pepsin) the ratio p_{305}/p_{270} was much reduced, approaching the values observed in the simple indole derivatives. The effect of urea was rapid. The polarizations were determined 30 min. after the addition of the urea. After 24 hr. at 4° the values remained the same in four proteins in which the time course was followed.

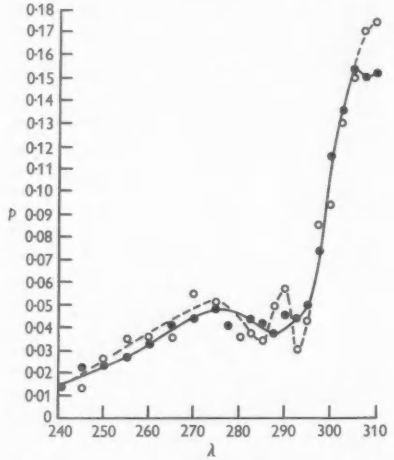

Fig. 11. Polarization spectra in 50% propylene glycol–water at −70°. ●, Chymotrypsin; ○, chymotrypsinogen.

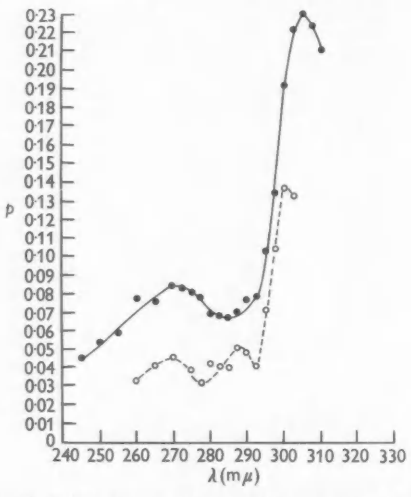

Fig. 12. Polarization spectra in 50% propylene glycol–water at −70°. ●, Pepsin; ○, fibrinogen.

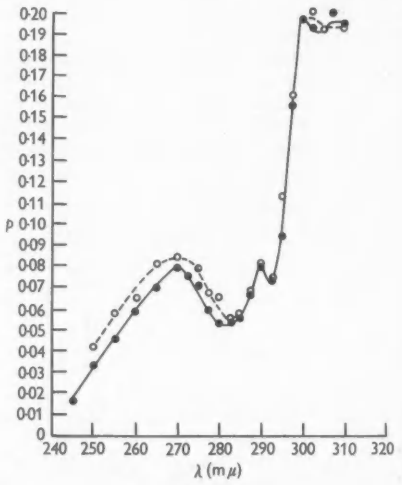

Fig. 13. Polarization spectra in 50% propylene glycol–water at −70°. ●, Bovine-serum albumin; ○, human-serum albumin.

Fig. 14. Polarization spectra of the fluorescence of proteins in buffer, pH 7·0. Proteins were dissolved in 8 M-urea and kept at 4° for 24 hr. before thorough dialysis against phosphate buffer, pH 7·0. ●, Bovine-serum albumin; ×, lactic dehydrogenase; ○, chymotrypsinogen.

(6) Three proteins were studied after removal of the urea. The polarization of the fluorescence was determined in the fraction that remained in solution after prolonged dialysis against neutral buffer. The proteins did not recover the polarization spectrum characteristic of the native protein, as can be seen in Fig. 14. The two main bands were less defined and the p_{305}/p_{270} ratio was much smaller than in the native proteins.

DISCUSSION

The depolarization of the fluorescence due to the Brownian rotation of the protein particle can be calculated when the rotational relaxation time of the particle and the actual lifetime of the excited state of the fluorescence τ are known (Weber, 1953). The natural lifetime of the excited state τ_e can be calculated from the integrated absorption of the electronic band of lowest frequency (e.g. Förster, 1951)

$$\frac{1}{\tau_e} \simeq 2{\cdot}88 \times 10^{-9} . \bar{\nu}^2 . n^2 \int_0^\infty \epsilon(\nu)\, d\nu.$$

From the Gaussian approximation already discussed (Weber, 1960) and data for phenol and tyrosine already published (Teale & Weber, 1957)

$$\tau_e \simeq 42\ \mu\text{m.sec.}$$

if the processes that decrease the quantum yield of tyrosine from unity are competitive with the emission, $\tau \simeq q\tau_e$. On this assumption the actual lifetime of phenol or tyrosine in water is 8 μm.sec. and that of insulin about 1·5 μm.sec. In the same manner calculation of the natural lifetime of indole gives $\tau_e \simeq 8\ \mu$m.sec. From the quantum yields observed in the class B proteins (F. W. J. Teale, in preparation) their actual lifetimes appear to be in the range (6) of 1–3 μm.sec. Thus the actual lifetime of the excited state of the fluorescence of proteins of either class A or B is too short to reveal changes in the relaxation time of the particles when the viscosity is increased (Weber, 1953). The polarization method may prove useful, however, in the determination of the rotational relaxation time of tyrosine and tryptophan peptides of molecular weight up to a few thousand.

The class A proteins, containing no tryptophan, show a polarization spectrum which can be interpreted unequivocally by comparison with that of tyrosine or phenol. The existence of transfer of the electronic-excitation energy among the tyrosine residues results in the proteins in smaller values of the polarization over the entire wavelength range, as compared with the polarizations observed in phenol or tyrosine solutions. Since the probability of transfer of the excited state is proportional to the lifetime of the excitation, other things being equal, it follows that transfer of energy from the tyrosines with higher quantum yields, and therefore longer lifetime, to those with smaller quantum yield and shorter lifetime will occur more often than the converse process, and will result in a smaller quantum yield than that expected in the absence of transfer. The process of transfer may then be partly responsible for the low yield of fluorescence observed in these proteins. In the extreme case in which the probability of transfer to a group with negligible quantum yield is very high the fluorescence of the protein may be almost completely quenched, as exemplified by transfer to the non-fluorescent haem in the haem proteins (Weber & Teale, 1959).

In class B proteins the interpretation of the findings is much more uncertain. The polarization spectrum shows considerable variation among the proteins, contrasting with the small changes in the absorption spectrum. The findings in the solutions in propylene glycol–water at $-70°$, where rotation of the tryptophan residues between excitation and emission appears extremely unlikely, show that some influence is at work in the proteins which reduces considerably the polarization observed on excitation by 270 mμ light and has little effect on the polarization of the fluorescence excited by 305 mμ. It appears tempting to suggest that energy transfer, particularly of the type observed among the indole molecules, with its dependence on the exciting wavelength, is responsible for the effect. Although this may be responsible for part of the observed effect, another possibility must be considered: the relative weights of the G–S_1 and G–S_2 transitions (Weber, 1960) in the indole nucleus will determine the relative values of the polarizations at 270 and 305 mμ. In the proteins, extension of the G–S_2 transition towards the shorter wavelength could explain the experimental findings. The spectral changes in the G–S_2 transition could occur as a result of the interaction of the indole nucleus with the highly polarizable protein backbone. The effect of urea would follow the breaking of hydrogen bonds maintaining the backbone structure. The changed relations with the backbone and the increased freedom of rotation of the tryptophan residues would be sufficient to explain the observed effects. Whatever the relative importance of these two factors, namely relative weight of the G–S_1 and G–S_2 transitions and electronic-energy transfer, there is little doubt that the fluorescence-polarization spectrum is an individual and characteristic property of the proteins studied, and is sensitive to changes in the secondary structure of the protein, in a characteristic manner. Though the interpretation of the observed effects is not unequivocal, their correlation with other structural properties of the

proteins offers a possible way to the final understanding of them.

SUMMARY

1. The polarization spectrum of insulin, ribonuclease and zein is qualitatively identical with that of tyrosine or cresol, but the lower absolute values of the principal polarization indicate the existence of energy transfer among the tyrosine residues.

2. The polarization spectrum of proteins containing tryptophan is similar to that of *N*-glycyltryptophan but differs from it in the lower values of the polarization in the 270 mμ region. The ratio of the polarization on excitation by 305 mμ and by 270 mμ (p_{305}/p_{270} ratio), which varies from 1·4 to 1·7 in the simple indole derivatives, is found to be greater than two in nine out of ten globular proteins studied.

3. The polarization spectrum in 50% propylene glycol–water at $-70°$ shows an increased p_{305}/p_{270} ratio (2·3–3·0), and the polarization spectrum in 8M-urea shows this ratio decreased to the range 1·4–1·9 in eight out of ten proteins studied.

4. The changes of proteins in urea are only partially reversible. The soluble fractions obtained in three proteins had a distinctly changed polarization spectrum with a lowered p_{305}/p_{270} ratio.

5. Though energy transfer among the tryptophan residues may play a part in the observed effects, it is believed that a change in the relative intensities of the G–S_1 and G–S_2 transitions in tryptophan can explain these effects equally well.

REFERENCES

Förster, T. (1951). *Fluoreszenz Organischer Verbindungen*, p. 186. Gottingen: Vandenhoeck und Ruprecht.

Teale, F. W. J. & Weber, G. (1957). *Biochem. J.* **65**, 467.

Teale, F. W. J. & Weber, G. (1959). *Biochem. J.* **72**, 15P.

Warner, R. C. (1954). In *The Proteins*, vol. 2, part A, p. 437. Ed. by Neurath, H. & Bailey, K. New York: Academic Press Inc.

Weber, G. (1953). *Advanc. Protein Chem.* **8**, 415.

Weber, G. (1954). *Trans. Faraday Soc.* **50**, 552.

Weber, G. (1960). *Biochem. J.* **75**, 335.

Weber, G. & Teale, F. W. J. (1959). *Disc. Faraday Soc.* **27**, 134.

Biochem. J. (1960) **75**, 352

Amino Acid-Activating Systems from Pig Liver

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A reaction between amino acids and adenosine triphosphate catalysed by specific enzymes has been termed amino acid activation by Hoagland (1955). This is considered to consist of the formation of an enzyme–amino acyl adenylate complex with release of pyrophosphate. The amino acid-activating systems further catalyse a reaction between amino acid and 'soluble' ribonucleic acid (Hoagland, Zamecnik & Stephenson 1957) to form an amino acyl–ribonucleic acid compound which is usually assumed to be formed by the transfer of the amino acyl adenylate to 'soluble' ribonucleic acid. Evidence for these two reactions has been summarized by a number of authors (Lipmann, 1958; Zamecnik, Stephenson & Hecht, 1958; Berg & Ofengand, 1958; Novelli, 1958). It is not yet clear whether these two reactions are catalysed by one or more enzymes, and purification of the system might help to elucidate this point. Moreover, these reactions have special interest as possible early stages in protein synthesis, and further elucidation of their function might be assisted by the availability of the purified enzymes. Pig liver was found by us to be a source of protein catalysing both reactions, and one from which the isolation and purification of these enzymes might be attempted.

In this paper we describe the isolation and simple properties of amino acid-activating preparations, which are essentially free of ribonucleic acid, including 'soluble' ribonucleic acid. This in no way impairs their ability either to catalyse amino acid-dependent exchange of pyrophosphate, or the transfer of [^{14}C]amino acids to added 'soluble' ribonucleic acid. We have compared these preparations with pH 5·0 fractions from the same liver and found them not dissimilar in their general behaviour.

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The removal of ribonucleic acid from these amino acid-activating preparations profoundly alters certain of their properties, in that the enzymes are no longer precipitable at pH 5·0. Addition of ribonucleic acid to the preparations restores this property, the activating enzymes becoming precipitable with some degree of purification. 'Soluble' ribonucleic acid from liver is more effective than yeast ribonucleic acid in these respects.

The observation that the amino acid-activating enzymes may be freed from 'soluble' ribonucleic acid without impairing the activation reaction, as measured by pyrophosphate exchange, was made during attempts to remove microsomal particles from pig-liver extracts without the use of high-speed centrifuging, a technique not practicable when 1–2 kg. of tissue is being processed. We found that the addition of protamine sulphate to the liver extracts caused the microsomes to aggregate, allowing them to be removed at relatively low centrifuge speeds. At the same time the 'soluble' ribonucleic acid was also removed. A preliminary account of this work has already been published (Finch & Hele, 1959).

MATERIALS AND METHODS

Chemicals. L-Isoleucine, L-valine, 2-amino-2-hydroxymethylpropane-1:3-diol (tris) and adenosine triphosphate (disodium salt; ATP) were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. ATP was neutralized to pH 7·2 (indicator paper) with $MgCO_3$, to give disodium magnesium ATP. Tris was titrated to the required pH with HCl. L-Leucine was purified in the laboratory. A sample of L-valine from Roche Biochemicals used in early experiments showed considerable quantities of other amino acids on chromatography. L-Cysteine HCl, glycine and one sample of glutathione were obtained from Hopkin and Williams Ltd. All other amino acids (laboratory grade), iodoacetamide and protamine sulphate (salmon) were obtained from British Drug Houses Ltd. The solutions of iodoacetamide were freshly prepared just before use. The protamine sulphate was dissolved with gentle warming to give a final concentration of 10 mg./ml., and the slightly acidic solution was not neutralized. Another sample of glutathione was obtained from the Schwartz Laboratories Inc. *p*-Chloromercuribenzoic acid (sodium salt) and protamine sulphate (herring) were supplied by L. Light and Co. Ltd. Coenzyme A was obtained from Pabst Laboratories. Yeast ribonucleic acid (RNA) was supplied by the California Corporation for Biochemical Research and was purified by precipitation with ethanol and dialysis against distilled water for 18 hr. Nu-char C activated charcoal, supplied by West Virginia Pulp and Paper Co., N.Y., U.S.A., was treated with 6N-HCl under vacuum for at least 2 hr., then washed with water until the washings were above pH 4·0 (indicator paper).

Radioactive tracers. Uniformly labelled L-[^{14}C]amino acids and $H_3{}^{32}PO_4$ in HCl were obtained from The Radiochemical Centre, Amersham, Bucks. The [^{14}C]amino acids were mixed with unlabelled amino acid for use at a specific activity of 4–5 μc/μmole. The $H_3{}^{32}PO_4$, as the monohydrogen salt, was converted into pyrophosphate by pyrolysis at 400° for 1 hr. (Lee Peng, 1956).

Preparations of amino acid-activating fractions

Fraction precipitated by ammonium sulphate–potassium acetate. Pig liver, obtained from T. Wall and Sons (London, N.W. 10), was taken directly from the animal within 10 min. of death (and not selected from the pooled offal) and packed in ice and conveyed to the Laboratory cold room, where work upon the tissue commenced within 30 min. of the death of the animal. All subsequent operations were carried out between 0° and 4°.

It was first necessary to carry out a pilot preparation upon a sample of the liver to ascertain the optimum amount of protamine sulphate needed to aggregate the microsomes. A portion (100 g.) of tissue was sliced, chopped with a heavy knife to give a thick pulp, stirred mechanically for 1 min. with 2 vol. of medium C (sucrose, 0·35M; $KHCO_3$, 0·035M; KCl, 0·025M) and filtered through cheese cloth, yielding a volume of extract about equivalent to the volume of medium used. Four 25 ml. samples of the extract were treated with protamine at levels ranging from 0·5 mg./ml. of extract to 2·0 mg./ml. Salmine sulphate was used, since herring protamine was found not to be effective. The protamine solution was added to the samples of extract over 1–2 min. with constant stirring; the samples were then centrifuged at 1880 *g* for 10 min., which sedimented the bulk of the particles. The concentration of protamine for treating the main preparation was chosen as slightly below the lowest level giving tight packing of the precipitate. This errs on the side of caution.

The main preparation, involving 1–2 kg. of liver, was carried out upon identical lines, 500 g. batches of tissue being used. If the concentration of protamine used in the main preparation proved insufficient to remove the particles completely, a further centrifuging at 10 000 *g* for 5–10 min. was performed in the Spinco model L preparative ultracentrifuge. The supernatant was treated with a mixture of solid $(NH_4)_2SO_4$ and potassium acetate (28 g. and 5 g./100 ml. of supernatant respectively) and spun at 10 000 *g* for 5–10 min. The precipitate, which comprised 25% of the protein, was dissolved in 0·01M-tris–HCl buffer, pH 7·2, and spun for 10–30 min. at 105 000 *g* to remove glycogen and small, variable amounts of particulate materials. The supernatant, referred to as ASAc fraction, was stored in small batches at −15°. Liver gave 2·5–3·5 g. of ASAc-fraction protein/500 g. The ASAc fractions used in the experiments upon the incorporation of [^{14}C]amino acids into 'soluble' RNA were dialysed for 2–4 hr. against 0·01M-tris–HCl buffer, pH 7·5, to remove some of the salts, and centrifuged at 105 000 *g* for 10–20 min. before use.

The ASAc fraction retained its pyrophosphate-exchange activity for at least 6 weeks if kept at −15°. Similar preparations, precipitated at 60% saturation with $(NH_4)_2SO_4$, either un-neutralized or neutralized with KOH or tris during the addition of the salt, gave better yields of threonine- and methionine-activating enzymes, but did not retain their pyrophosphate-exchange activity for more than 2 or 3 days.

The ASAc fraction contains about 0·1–0·3 mg. of RNA/50 mg. of protein/ml. The lower value is just detectable by our methods. This detectable RNA can be completely

removed by centrifuging at 105 000 *g* for 10 min., without affecting either the pyrophosphate-exchange rate or the labelling of added 'soluble' RNA.

Rat-liver ASAc fractions were made from minced tissue along lines identical with those used with pig liver. Their properties were very similar to those of the pig-liver preparations.

We found that the effective precipitation of microsomal particles with protamine sulphate could be achieved only if liver extracts were prepared in the manner described above. Extracts made with phosphate buffers, extracts in which the ratio of tissue to medium C was doubled or homogenates prepared in Waring-type blenders required more protamine sulphate to remove the particles and the ensuing particle-free extracts contained very little activating-enzyme activity.

We suggest that the success of this method for the removal of particles may depend upon a lowered ratio of particulate elements to soluble protein. Compared with homogenates prepared in a blender, the chopping and extracting procedure described above releases about 50% of the soluble protein, 25% of the RNA and only 15–20% of the particulate material, including glycogen. The soluble protein obtained from both chopping and homogenizing gives pH 5·0 fractions with the same specific activity for amino acid-dependent exchange of pyrophosphate, tested with 18 amino acids separately.

We observed that if the cheesecloth filtrate was allowed to stand for more than a few minutes without the addition of protamine, then virtually all amino acid-activating activity was lost. This did not happen if protamine were added immediately after the preparation of the filtrate. In practice this required that the pilot run should be commenced before the main bulk of the material was chopped.

Fraction precipitated at pH 5·0. Preparations were made from the livers of young Wistar rats, weight 80–100 g., by the method of Keller & Zamecnik (1956), but with adjustment of the supernatant from the centrifuging for 2 hr. at 105 000 *g* to pH 5·0 rather than pH 5·2. Similar preparations were made from homogenized pig liver and from the cheesecloth-filtered extract of chopped pig liver. For all of the fractions precipitated at pH 5·0 (pH 5·0 fractions) the tissues were dispersed in medium A (Keller & Zamecnik, 1956), from which our medium C is derived by omission of $MgCl_2$.

Isolation of 'soluble' ribonucleic acid. 'Soluble' RNA was isolated from the pH 5·0 fractions by the phenol method of Kirby (1956) as described by Hoagland, Stephenson, Scott, Hecht & Zamecnik (1958).

Assays of enzyme activity

Pyrophosphate exchange. For the standard assay a mixture of 1 μmole of amino acid, 6 μmoles of disodium magnesium ATP, 3 μmoles of [^{32}P]pyrophosphate containing 20 000–50 000 counts/min., 100 μmoles of tris–HCl buffer, pH 7·2, and 1–2 mg. of protein in a total volume of 1 ml. was incubated in 15 ml. centrifuge tubes at 38° for 5 min. without shaking, and then treated with 3·5 ml. of ice-cold 6% trichloroacetic acid. Amino acid was omitted from the control incubation. After centrifuging in the cold to remove the protein precipitate, the supernatant was decanted on to 0·4 ml. of moist charcoal (approx. 160 mg. dry wt.) for the separation of ATP and pyrophosphate by a modification of the method of Crane & Lipmann (1953). The modification consisted of using filtration instead of centrifuging for the washing of the charcoal, in a microfilter tube (A. Gallenkamp Ltd., no. AG 12A × 4; capacity 8 ml.) with the outlet stoppered with a polyvinyl chloride cap. The filter tube was fitted into a receiving vessel with a side arm. (Filter tube with side arm, 150 mm. × 25 mm. diam., capacity 40 ml.; Griffin and George Ltd., no. S25–720.) Layering with ethanol was unnecessary. With a manifold connexion to the vacuum pump up to forty incubations could be treated at one time. The arrangement of the filter tube and receiving vessel is shown in Fig. 1. Charcoal was measured into the filter tube with a sawn-off pipette fitted with a glass tube as a plunger. The radioactive samples were added to the charcoal and mixed with glass rods. The filter tubes were capped to prevent loss of liquid before adsorption of ATP had taken place. After a brief interval, when all the samples had been well mixed with the charcoal, the caps were removed and suction was applied through a manifold. The charcoal was washed with water up to the 15 ml. graduation on the receiving vessel. This solution was on occasion retained for estimation of liberated orthophosphate. Suction was stopped, the filter tube and top stopper were removed together and the cap was replaced on the bottom of the filter tube. The charcoal was stirred with 2 ml. of 2N-HCl and the filter tube, top stopper and cap were transferred to a boiling-water bath for 15 min. to hydrolyse the labile phosphates of ATP. At the end of this period the caps were removed from the

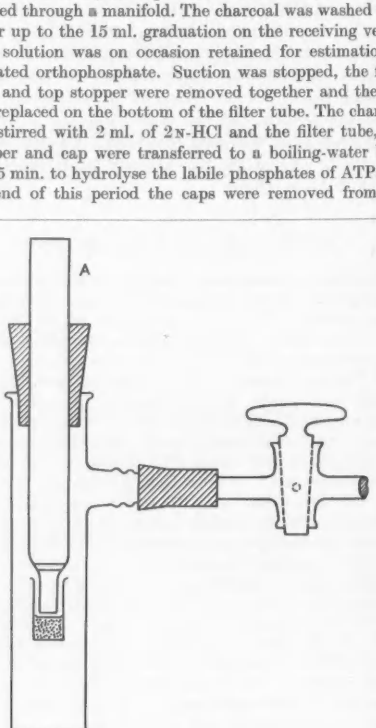

Fig. 1. Filtration equipment used for washing charcoal precipitates. A, Filter tube, top stopper and cap; B, receiving vessel, graduated at 15 ml.

bottom of the filter tubes and the filter tubes and top stoppers were quickly returned to the receiving vessels, which had meantime been washed out six times with water and dried in an oven. Suction and washing were applied as before, resulting in a 15 ml. sample suitable for estimation of radioactivity and orthophosphate (P_i). After correction for the recovery of P_i from ATP (usually 80–90%) the rates of exchange were calculated according to Davie, Koningsberger & Lipmann (1956).

The amino acid-dependent exchange of pyrophosphate was taken as the difference between test and control incubations. All our data on pyrophosphate exchange are expressed after the subtraction of the control, except where specifically stated otherwise.

The rate of amino acid activation is influenced by the amount of pyrophosphate in the system (Davie *et al.* 1956). P_i found (about 0·8 μmole) in the first washings from the charcoal indicates a maximum loss of pyrophosphate of 10%.

Attachment of [^{14}C]*amino acids to* '*soluble*' *ribonucleic acid.* A mixture of 0·04 μmole of [^{14}C]amino acid, 4 μmoles of disodium magnesium ATP, about 100 μg. of 'soluble' RNA and about 4 mg. of protein in a total volume of 0·4 ml. was incubated in 15 ml. centrifuge tubes at 38° for 4 min. without shaking and then treated with 1 ml. of ice-cold 0·6 N-perchloric acid, followed by centrifuging at 1880 *g* in the cold. Controls were performed with the omission of either ATP or 'soluble' RNA as appropriate. The supernatant was discarded and the precipitate washed with cold 0·2 N-perchloric acid and lipid solvents according to the procedure of Hoagland *et al.* (1958). In some experiments the RNA was extracted from the washed precipitate with hot NaCl by the method of these authors, but more generally the total precipitate was dissolved by warming in 0·1 ml. of N-NaOH followed by 0·2 ml. of water. Volumes of 0·1 ml. were taken for plating and counting and for estimation of RNA (ribose) content. In certain control experiments the labelled RNA was extracted from the total precipitate with hot 0·8 N-perchloric acid before counting (Rendi & Campbell, 1959). 'Soluble' RNA recoveries in the samples used for counting rarely differed by more than 25% within any one experiment, and agreement between samples was usually of the order of 10–15%.

Measurement of radioactivity

^{32}P was counted in a M. 6 liquid counter of 9 ml. capacity (20th Century Electronics Ltd.) and corrected for the decay of the isotope.

Materials containing ^{14}C were plated as thin samples on lens paper in 2 cm.^{2} polythene planchets and counted with an end-window counter. The counts were corrected to negligible thickness by the use of an experimentally determined self-absorption curve. The maximum corrections thus introduced were less than 10%.

Owing to the large numbers of samples that had to be counted the time of counting of any one specimen was restricted to 5 min. The standard error of counting of weakly radioactive specimens was as high as ± 15%, but for more active specimens this ranged between ± 3 and ± 8%. The background counting rate was always determined to an accuracy of ± 5%, generally being $10 \pm 0{\cdot}5$ counts/min.

In the studies with both pH 5·0 fraction and our 'recombined system' the control experiments, from which either ATP or added 'soluble' RNA was omitted, seldom gave counting rates higher than the background count. The highest control count observed on one occasion only was 3 counts/min. above background. Experiments with complete systems gave counting rates of between 10 and 250 counts/min. above the background count. Counting rates of 5 counts/min. above background or less were of little significance.

We have expressed our experimental findings on the labelling of 'soluble' RNA in terms of μm-moles of amino acid incorporated/mg. of 'soluble' RNA. This allows us to correct for the varying specific activities of our [^{14}C]amino acid solutions, and for the amount of RNA used in the assay of radioactivity. This latter correction is important, for although the recoveries of RNA were very consistent within any one experiment, wide variations occurred between experiments. Standard errors for these corrected values were calculated from the standard errors of counting the samples.

Chemical estimations

RNA was estimated as ribose by the orcinol method of Albaum & Umbreit (1947), the colour being developed by heating for 40 min. in stoppered tubes in a boiling-water bath. ATP was used as a ribose standard, since ribose itself or adenosine monophosphate gave poorly reproducible results. The results were expressed as RNA on the basis of a conversion factor of 670 mg. of RNA $\equiv$ 1 μmole of ATP ribose, determined for a standard preparation of RNA. This standard preparation was isolated from mouse-ascites-tumour cells by the method of Davidson & Waymouth (1944) and contained 8·1% of P.

Determinations of the RNA content of enzyme preparations or tissue fractions were carried out on the precipitate of denatured protein obtained with 0·8 N-perchloric acid and washed with 0·2 N-perchloric acid and lipid solvents as in the assay for attachment of [^{14}C]amino acids to 'soluble' RNA. The estimation was carried out on either the extract obtained by overnight incubation at 38° with 0·8 N-perchloric acid or on the solution in NaOH as used for plating and counting. The estimations by these two procedures showed good agreement. When the precipitates from perchloric acid contained large amounts of protein relative to the amount of RNA present (less than 200 μg. of RNA/100 mg. of protein) a brown or yellow colour often developed during the boiling of the orcinol reagents, which interfered with measurements of E at 660 mμ. This made it very difficult to detect small quantities of RNA with any certainty. For this reason we were unable to detect with confidence small amounts of 'soluble' RNA added to ASAc-fraction protein (Fig. 6), and for this reason we considered all values below 5 μg. of RNA found in the portions taken for plating and counting to be of little significance.

Like Simpkin & Work (1957) we have found that microsomes often lose a considerable quantity of their RNA in the presence of cold dilute perchloric acid. This loss can be avoided by precipitating the microsomes with protamine, resuspending and then reprecipitating them with cold perchloric acid and washing in the usual manner.

Protein was determined usually by the biuret procedure of Gornall, Bardawill & David (1949) and on occasion by the turbidimetric method used by Hoagland, Keller & Zamecnik (1956) and by Stadtman, Novelli & Lipmann (1951). These three methods agreed to within 10%.

Sulphydryl groups were determined by the procedure of Grunert & Phillips (1951) and P_i by the method of Allen (1940).

RESULTS

Removal of particles with protamine

The contents of some constituents of the supernatant obtained after treatment with varying amounts of protamine are given in Fig. 2. The ribonucleic acid content of the supernatant drops rapidly, consistent with the observed loss of microsomal particles. The turbidity in the turbidimetric estimation (the value measured deriving both from soluble protein and from particulate material) drops initially in a similar manner, reaching a constant low level which represents largely a mixture of soluble protein and glycogen. With concentrations of protamine above that necessary to bring down the bulk of the ribonucleic acid and the particles, a loss of activating enzymes as obtained in the ASAc fraction is observed. We do not know the extent to which the upward trend in activity shown in this curve is maintained at concentrations of protamine above 2·0 mg./ml. of extract, although early experiments with higher concentrations of protamine than those employed here did not give very encouraging results in terms of enzyme activity. No significant decrease in –SH occurs over this range of protamine concentrations.

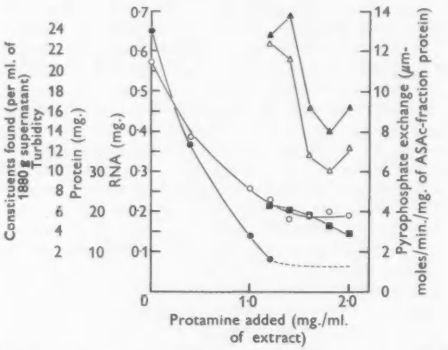
Constituents found (per ml. of 1880 g supernatant)
Turbidity
Protein (mg.)
RNA (mg.)
Pyrophosphate exchange (μm-moles/min./mg. of ASAc-fraction protein)
Protamine added (mg./ml. of extract)

Fig. 2. Removal of particulate material and amino acid-activating enzymes from a pig-liver extract treated with protamine sulphate. Increasing amounts of a protamine sulphate solution (10 mg./ml.) were added, with stirring, over 1 min. to 50 ml. samples of a pig-liver extract prepared as described under Materials and Methods. The samples were centrifuged for 10 min. at 1880 *g* and determinations made upon the supernatants so obtained of RNA content (●), turbidity by the method of Hoagland, Keller & Zamecnik (1956), the results being given here in arbitrary turbidity units/ml. (○) and protein by the biuret procedure (■). Amino acid-dependent exchange of pyrophosphate was measured upon samples of ASAc fractions prepared from the 1880 *g* supernatants by the assay described under Materials and Methods, with leucine (△) and an impure sample of valine (▲) as substrates, the latter containing isoleucine and methionine. Very small amounts of RNA could not be detected accurately (see Materials and Methods). The broken line indicates the amount assumed to be present.

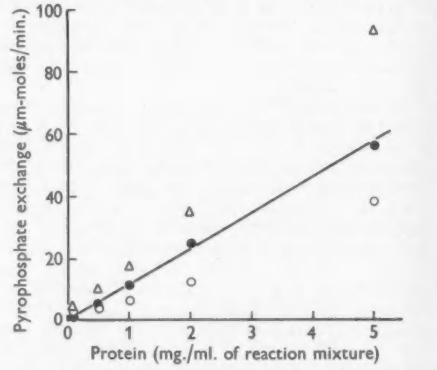

Fig. 3. Effect of protein concentration upon the rate of isoleucine-dependent exchange of pyrophosphate catalysed by a pig-liver ASAc fraction. The standard assay, as described under Materials and Methods, was used with varying amounts of protein as indicated. The ASAc fraction was dialysed for 2 hr. before use. △, Total pyrophosphate exchange; ○, amino acid-free pyrophosphate exchange; ●, amino acid-dependent pyrophosphate exchange, shown as the difference between the first two determinations.

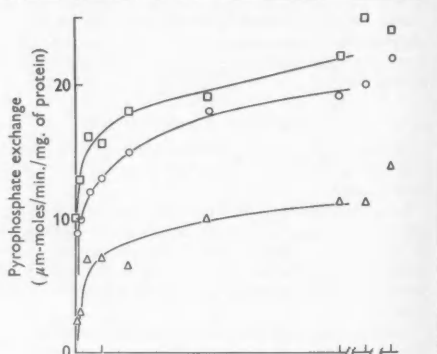

Fig. 4. Effect of amino acid concentration upon the rate of amino acid-dependent exchange of pyrophosphate catalysed by a pig-liver ASAc fraction. The standard assay, as described under Materials and Methods, was used. The ASAc fraction was dialysed for 4 hr. before use and 2·1 mg. of protein was used in the incubation mixture. □, L-leucine; ○, L-isoleucine; △, L-valine.

Amino acid-dependent exchange of pyrophosphate catalysed by pig-liver preparations

Without dialysis the ASAc fraction gave high control values in the pyrophosphate-exchange assay in the absence of added amino acids. Dialysis against 0·01 M-tris–HCl buffer, pH 7·2, for 2–3 hr. reduced the control values to the order of 10–20 μm-moles/mg. of protein/min. The rate of amino acid-dependent exchange of pyrophosphate remained linear with time for at least 5 min. With the dialysed preparations the amino acid-dependent exchange of pyrophosphate was linear with protein concentration to at least 5 mg. of protein (Fig. 3). For L-isoleucine, L-leucine and L-valine the rate increases with concentration of amino acid as shown in Fig. 4, with half-maximal rates being reached in the range 0·5–0·1 mM. The rate at the standard-assay concentration of mM was about three-quarters of the maximum for these amino acids. No inhibition was observed at high concentrations.

The ASAc fraction and the pH 5·0 fraction predominantly activate leucine and isoleucine (Table 1). The activities of the pH 5·0 fractions from the same liver are usually 20–50 % higher than those of the ASAc fraction. The rat-liver fractions are two to three times as active as the comparable pig-liver ones. With the majority of preparations the values of the amino acid-dependent exchange of pyrophosphate were higher than those in Table 2, but the relative order of the rates remained much the same.

The addition of 'soluble' RNA to incubations of ASAc fractions did not influence the amino acid-dependent exchange of pyrophosphate (Table 2), even if pre-incubated with the protein. In some cases a considerable reduction of the exchange in the amino acid-free control has been observed. Yeast RNA at a concentration of 0·5 mg./ml. has no effect on the pyrophosphate exchange.

The ASAc fraction contains about 0·3 μmole of SH/ml. (20–50 mg. of protein) as determined upon boiled extracts, or upon cold perchloric acid extracts. Periods of dialysis up to 18 hr. had no effect on the amino acid-dependent exchange of pyrophosphate, although decreasing the blank. The presence of 0·05 M-glutathione, mM-ethylenediaminetetra-acetate or 0·01 M-$MgCl_2$ in the dialysis buffer did not affect the amino acid-dependent rate. The addition of glutathione, coenzyme A and acetate was without effect on the level of pyrophosphate exchange. The amino acid-dependent rate in the standard assay was reduced to 20 % for both ASAc and pH 5·0 fractions in the presence of 0·1 mM-*p*-chloromercuribenzoic acid and was unchanged and reduced to 50 % respectively in the presence of 10 mM-iodoacetamide.

Effect of ribonucleic acid on the precipitation of amino acid-activating enzymes at pH 5·0

Adjustment of pH to 5·0 does not cause precipitation from the dialysed ASAc fraction, provided that it was centrifuged after dialysis. If 'soluble'

Table 1. *Typical rates of amino acid-dependent exchange of pyrophosphate catalysed by pig-liver preparations*

The standard assay, as described under Materials and Methods, was used.

Amino acid	Pyrophosphate exchange (μm-moles/min./mg. of protein) ASAc fraction	pH 5·0 fraction
Alanine	0	0
Arginine	0	1
Asparagine	0	1
Aspartic acid	0	5
Glutamic acid	0	5
Glycine	4	1
Histidine	0	7
Isoleucine	21	22
Leucine	17	18
Lysine	0	2
DL-Methionine	6	10
Phenylalanine	2	0
Proline	4	7
Serine	4	4
DL-Threonine	2	5
Tryptophan	6	5
Tyrosine	0	4
Valine	7	6

Table 2. *Effect of added 'soluble' ribonucleic acid upon the rate of amino acid-dependent exchange of pyrophosphate catalysed by the 'ASAc fraction'*

The standard assay as described under Materials and Methods was used.

In Expt. 1 the ASAc fraction was dialysed for 2 hr. against 0·01 M-tris buffer, pH 7·2, and then centrifuged for 10 min. at 105 000 *g*; 0·75 mg. of protein was used and 0·75 mg. of 'soluble' RNA was added as indicated.

In Expt. 2 the ASAc fraction was dialysed for 2·5 hr. against the same buffer and then pre-incubated for 3·5 min. at 37° with added 'soluble' RNA (2·3 mg./100 mg. of protein). The pre-incubated mixture was cooled in ice for 2·5 min. and then tested in the standard assay.

All data obtained with added amino acids are expressed as the excess over the amino acid-free blank.

	Pyrophosphate exchange (μm-moles/min./mg. of protein) Without added 'soluble' RNA	With added 'soluble' RNA
Expt. 1		
No amino acid	20	18
Leucine	14·7	14·7
Isoleucine	21·4	24·2
Expt. 2		
No amino acid	21	12·8
Leucine	7	7·4
Isoleucine	8·7	6

RNA or yeast RNA be added before adjusting the pH to 5·0, then the lowering of pH causes precipitation, the amount of protein precipitated being proportional to the amount of RNA added.

This precipitation results in a purification of some of the amino acid-activating enzymes. The optimum ratio of protein to RNA giving the best increase in specific activity of the enzyme appears to be about 50:1; the specific activity increases with the ratio up to this point, and thereafter decreases. The same value for the optimum ratio is found with both yeast RNA and 'soluble' RNA but 'soluble' RNA gives a sixfold purification, as compared with the threefold purification obtained with yeast RNA (Table 3). About twice as much 'soluble' RNA as yeast RNA is precipitated at and below the ratio (50:1) of RNA to protein, although the amount of protein precipitated is the same (Table 4). This relatively lower yield of yeast RNA to protein in pH 5·0 precipitates has been observed in a number of experiments.

Table 3. *Purification of amino acid-activating enzymes in the 'ASAc fraction' by precipitation at* pH 5·0 *in the presence of added ribonucleic acid*

ASAc fraction was diluted to a concentration of 1 mg. of protein/ml. with 0·01 M-tris–HCl buffer, pH 7·5, and yeast RNA or 'soluble' RNA was added in the proportion of 2 mg. of RNA to 100 mg. of protein. pH 5·0 fractions were prepared from these mixtures as described under Materials and Methods and assayed in our standard system for pyrophosphate exchange.

	Pyrophosphate exchange (μm-moles/min./mg. of protein)	
	ASAc fraction	pH 5·0 fraction
Expt. 1 (with 'soluble' RNA)		
Isoleucine	16·0	122
Leucine	20·4	124
Expt. 2 (with yeast RNA)		
Isoleucine	15·0	48

'pH 5·0 *Fraction*' *prepared from pig liver and rat liver*

Pig-liver pH 5·0 fraction does not differ greatly from rat-liver pH 5·0 fraction in its ability to incorporate [^{14}C]amino acids into 'soluble' RNA (Table 5). A far greater difference exists between the rates of amino acid-dependent exchange of pyrophosphate catalysed by the pH 5·0 fractions from the two different sources. Two pig-liver pH 5·0 fractions tested with [^{14}C]phenylalanine and [^{14}C]lysine gave values for incorporation of the same order as valine and threonine. [^{14}C]Tryptophan was not incorporated. The value for [^{14}C]-

Table 4. *Precipitation of protein and ribonucleic acid at* pH 5·0

ASAc fraction was dialysed for 3 hr. against 0·01 M-tris–HCl buffer, pH 7·5, and then centrifuged at 105 000 *g* for 15 min. Samples of this protein (50 mg.) were diluted with tris buffer to a final concentration of 2 mg./ml., and RNA was added in the quantities indicated in the Table. pH 5·0 fractions were prepared as described in the Materials and Methods section. No precipitation occurred in the absence of added RNA.

Total quantity found in pH 5·0 fraction	'Soluble' RNA				Yeast RNA			
	RNA added (mg./100 mg. of protein)							
	1	2	3	4	1	2	3	4
Protein (mg.)	8·1	14·2	20·2	24·2	7·8	14·8	22·4	24·8
RNA (mg.)	0·15	0·26	0·26	0·26	0·07	0·13	0·28	0·29

Table 5. *Incorporation of* [^{14}C]*amino acids into 'soluble' ribonucleic acid and the amino acid-dependent exchange of pyrophosphate cataylsed by pig- and rat-liver* pH 5·0 *fraction*

The assay systems described under Materials and Methods were used.

	Amino acid incorporated into 'soluble' RNA (μm-moles/mg./4 min.)			
	Leucine	Isoleucine	Valine	Threonine
Pig pH 5·0 fraction	2·2±0·17*	6·7±0·69	0·74±0·12	1·1±0·15
Rat pH 5·0 fraction	4·3±0·18	7·2±0·21	0·60±0·15	1·0±0·08
	Pyrophosphate exchange (μm-moles/mg. of protein/min.)			
	Leucine	Isoleucine	Valine	Threonine
Pig pH 5·0 fraction	24·3	28·7	6·1	7·4
Rat pH 5·0 fraction	90·0	152·0	—	17·5

* S.E. derived from standard error of ^{14}C counting.

valine obtained with the rat-liver preparation is abnormally high. In most cases essentially zero incorporation was obtained with rat-liver pH 5·0 fractions with [^{14}C]valine.

Incorporation of [^{14}C]*amino acids into added 'soluble' ribonucleic acid catalysed by 'recombined' systems*

ASAc fractions of low RNA content easily transfer [^{14}C]amino acids to added 'soluble' RNA, previously isolated by phenol extraction from pH 5·0 fractions (see Materials and Methods section). We term such a system a 'recombined' system. ASAc fractions over 6 weeks old are as effective as freshly prepared material, but solutions of isolated 'soluble' RNA deteriorate after about 3 days, and the best results with 'recombined' systems are obtained with freshly isolated 'soluble' RNA. The extent of incorporation of [^{14}C]amino acid into the added 'soluble' RNA in the 'recombined' system is usually comparable, although not identical, with the incorporation obtained with a sample of the pH 5·0 fraction from which the 'soluble' RNA was originally isolated (Table 6). The rate of pyrophosphate exchange, and therefore of the potential quantity of acyl amino acid formed, is far greater than the rate of incorporation of amino acid into 'soluble' RNA, or of the total quantity of labelled 'soluble' RNA formed. As we noted with the pH 5·0 fractions, there was no clear relationship between the abilities of our various systems to catalyse the two reactions. Thus in Expt. 1 (Table 6) the total amount of valine adenylate potentially generated by the rat-liver pH 5·0 fraction amounts to 680 μm-moles, by the rat-liver ASAc fraction 1200 μm-moles and by the pig-liver ASAc fraction 280 μm-moles. In Expt. 2 the rat-liver pH 5·0 fraction would have produced 20 000 μm-moles of leucine adenylate during the period of incubation and the pig-liver ASAc fraction 2900 μm-moles.

In several cases we observed that more [^{14}C]-valine, and possibly more [^{14}C]threonine, was incorporated into 'soluble' RNA acid in the 'recombined' system consisting of pig-liver ASAc fraction +rat-liver 'soluble' RNA than occurred with the

Table 6. *Incorporation of* [^{14}C]*amino acids into added 'soluble' ribonucleic acid in a 'recombined' system*

The assay system described under Materials and Methods was used. In Expt. 4 the final volume was 0·5 ml. and the incubation time 5 min., and the 'soluble' RNA and protein were pre-incubated together for 4 min. at 37° before the addition of [^{14}C]amino acid and ATP. The pH 5·0 fractions were samples of those from which the 'soluble' RNA appropriate to the experiment had been isolated. Protein and 'soluble' RNA were added in the quantities indicated. Two different pig ASAc fractions were used: (*a*) and (*b*).

	Amino acid incorporated into 'soluble' RNA (μm-moles/mg./4 min.)			
	Leucine	Isoleucine	Valine	Threonine
Expt. 1				
Rat pH 5·0 fraction (120 μg. of 'soluble' RNA + 4·1 mg. of protein)	1·5±0·22*	9·8±0·43	0	0·9±0·11
Rat 'soluble' RNA (155 μg.; 1-day-old) + rat ASAc (3·9 mg. of protein)	2·0±0·21	4·2±0·34	1·0±0·16	1·4±0·21
Rat 'soluble' RNA (155 μg.; 1-day-old) + pig ASAc (*a*) (3·9 mg. of protein)	2·2±0·21	2·7±0·23	1·7±0·22	1·1±0·21
Expt. 2				
Rat pH 5·0 fraction (45 μg. of 'soluble' RNA + 2·25 mg. of protein)	59·±0·51	12·3±0·89	0	1·4±0·26
Rat 'soluble' RNA (63 μg.; 1-day-old) + pig ASAc (*b*) (4·0 mg. of protein)	6·3±0·67	10·5±0·76	1·4±0·35	2·3±0·21
Expt. 3				
Pig pH 5·0 fraction (89 μg. of 'soluble' RNA + 5·5 mg. of protein)	4·4±0·26	5·8±0·26	1·8±0·12	1·1±0·09
Pig 'soluble' RNA (190 μg.; 4-day-old) + pig ASAc (*b*) (4·2 mg. of protein)	1·8±0·09	—	1·3±0·10	0·8±0·09
Expt. 4 (incubation for 5 min.)				
Rat pH 5·0 fraction (240 μg. of 'soluble' RNA + 4·8 mg. of protein)	2·8±0·10	—	0·3±0·05	—
Rat pH 5·0 fraction, as above + 100 μg. of rat 'soluble' RNA	2·6±0·08	—	0·5±0·05	—
Rat 'soluble' RNA (100 μg.) + pig ASAc (*b*) (9·8 mg. of protein)	2·1±0·18	—	1·6±0·20	—

* S.E. derived from standard error of ^{14}C counting.

Table 7. *Incorporation of [^{14}C]amino acid into 'soluble' ribonucleic acid and protein in experiments with 'recombined' systems*

Duplicate samples of ^{14}C-labelled precipitates from perchloric acid were prepared as described in the Materials and Methods section, and one set of precipitates were dissolved in NaOH and counted. Where indicated, the duplicate precipitates were either extracted with hot 10% NaCl and the isolated 'soluble' RNA in this extract was counted, or they were extracted with hot 0·8 N-perchloric acid, and the residual precipitate (i.e. minus 'soluble' RNA) was dissolved in NaOH and counted.

	Amino acid incorporated into 'soluble' RNA (μm-moles/mg./4 min.)	
Expt. 1	'Total' perchloric acid precipitate	'Soluble' RNA from NaCl extract
Pig pH 5·0 fraction + Leucine	2·9 ± 0·18*	2·7 ± 0·27
Pig pH 5·0 fraction + Isoleucine	4·0 ± 0·20	3·7 ± 0·29
Pig ASAc + pig 'soluble' RNA + Leucine	1·8 ± 0·11	2·2 ± 0·21
Pig ASAc + pig 'soluble' RNA + Valine	1·3 ± 0·10	1·5 ± 0·15
Expt. 2	'Total' perchloric acid precipitate	'Total' perchloric acid precipitate after extraction with 0·8 N-perchloric acid
Pig ASAc + rat 'soluble' RNA + Isoleucine	6·7 ± 0·31	0·0
Pig ASAc + rat 'soluble' RNA + Valine	1·4 ± 0·17	0·0

* S.E. derived from standard error of ^{14}C counting.

appropriate rat-liver pH 5·0 fraction. These effects, although small, represented a significant amount of labelling; indeed, no labelling at all occurred with some of the rat-liver pH 5·0 fractions.

This increased incorporation of [^{14}C]valine might possibly be attributed to the presence of microsomes in the ASAc fraction, which would lead to the incorporation of label into microsome protein, as well as into 'soluble' RNA. These effects would not be differentiated when 'total' perchloric acid precipitates were used for the estimation of radioactivity. Control experiments (Table 7), designed to test this point, showed that 'soluble' RNA could be extracted with hot 10% NaCl from the 'total' perchloric acid precipitate, and that this extracted RNA was labelled to the same extent as that in the 'total' precipitate. Moreover, RNA could be completely extracted from the 'total' precipitate (under conditions that would have left labelled protein behind in the 'total' precipitate) and with it all the radioactivity of the 'total' precipitate. It is unlikely that the increase in [^{14}C]valine incorporated described above was stimulated by the small quantity of –SH in the ASAc fraction (cf. Rendi & Campbell, 1959).

Some simple studies of the kinetics of the 'recombined' system have been made. The rate of incorporation of [^{14}C]isoleucine is proportional to the concentration of ASAc-fraction protein (Fig. 5*b*), when measured on the linear portion of the time curve (Fig. 5*a*). The amount of [^{14}C]leucine and [^{14}C]valine incorporated into added 'soluble' RNA is proportional to the amount of 'soluble' RNA added, for an incubation period of 4 min. (Fig. 6).

DISCUSSION

Exchange of pyrophosphate

Pig-liver preparations catalyse the activation of much the same spectrum of amino acids as do other mammalian-liver preparations, and, broadly speaking, at much the same rates (cf. Novelli, 1958). The results of the amino acid-concentration studies indicate the affinities of L-isoleucine, L-leucine and L-valine for their activating systems to be similar to those found for L-tryptophan (Davie *et al.* 1956), L-tyrosine (Schweet & Allen, 1958) and L-methionine (Berg, 1956).

The relative rates of activation of the various amino acids by both the ASAc fraction and pH 5·0 fraction are similar to those of Webster (1959) observed with pig-liver preparations. It is possible that the small amino acid-dependent exchange of pyrophosphate observed for many of the amino acids might be greater if tested at the optimum pH as proposed by this author.

That amino acid activation occurs in the absence of 'soluble' ribonucleic acid and is not stimulated by its addition is in line with the findings of Schweet & Allen (1958) for the tyrosine-activating enzyme from hog pancreas, and by the findings of Schweet, Bovard, Allen & Glassman (1958) for activating enzymes from guinea-pig liver. Our findings are also in line with those of Hoagland *et al.* (1958) and of Decken & Hultin (1959), who showed that treatment of pH 5·0 fractions with ribonuclease did not affect amino acid activation as measured by pyrophosphate exchange, thus differing from the findings of Ogata & Nohara (1957*a*, *b*).

Unlike Rendi, Di Milia & Fronticelli (1958) we can find no evidence that ribonucleic acid is inhibitory to amino acid activation, at least when this reaction is measured by following pyrophosphate exchange.

Precipitation at pH 5·0 *in the presence of ribonucleic acid*

Evidently the amino acid-activating enzymes require the presence of a polyanion before they become insoluble at acid pH, and any type of ribonucleic acid will serve this function. The greater degree of purification obtained with 'soluble' ribonucleic acid as compared with yeast ribonucleic acid suggests that the 'soluble' ribonucleic acid is more selective for the enzyme proteins. It is tempting to associate the twofold efficiency of 'soluble' ribonucleic acid (as compared with yeast ribonucleic acid) in the purification of these enzymes with the fact that twice as much 'soluble' ribonucleic acid is precipitated in the pH 5·0 fraction as is yeast ribonucleic acid. We do not know if it is simply the size of the ribonucleic acid molecule or its specific structure which decides this selectivity, for we have no information about the molecular size of the ribonucleic acids used in these experiments. Rendi & Campbell (1959) have shown that the protamine-treated 'S-protein' of Sachs (1957), of low ribonucleic acid content, is non-precipitable at pH 5·0.

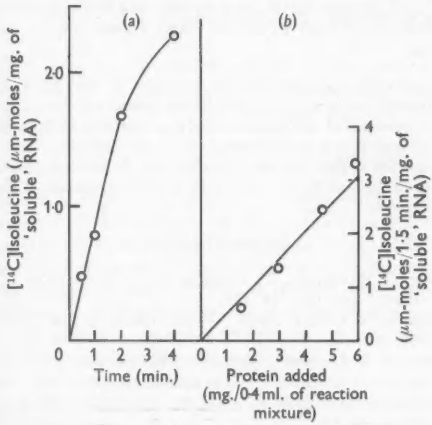

Fig. 5 (*a*). Effect of incubation time upon the incorporation of [^{14}C]isoleucine into 'soluble' ribonucleic acid in a 'recombined' system. The assay system described under Materials and Methods was used and contained 3·85 mg. of pig ASAc fraction and 155 μg. of rat-liver 'soluble' RNA (5-day-old). A zero-time sample of the complete system gave the same radioactivity as the background count of 9 counts/min. Isoleucine gave a rate of amino acid-dependent exchange of pyrophosphate of 16 μm-moles/min./mg. of ASAc protein. The standard errors for these four points, calculated from the standard error of the ^{14}C counting, and reading from left to right, are $\pm$0·12, $\pm$0·13, $\pm$0·09, $\pm$0·12 μm-mole.

(*b*) Effect of ASAc-fraction protein concentration upon the rate of incorporation of [^{14}C]isoleucine into 'soluble' ribonucleic acid in a 'recombined' system. The assay system described under Materials and Methods was used and contained 210 μg. of rat-liver 'soluble' RNA (1-day-old) and pig-liver ASAc fraction in the amounts indicated. The incubation time was 1·5 min. The ASAc fraction gave a rate of amino acid-dependent exchange of pyrophosphate of 12 μm-moles/min./mg. of protein with isoleucine. The standard errors for these four points, calculated from the standard error of the ^{14}C counting, and reading from left to right, are $\pm$0·09, $\pm$0·09, $\pm$0·11, $\pm$0·19 μm-mole.

Attachment of amino acids to 'soluble' ribonucleic acid and its relation to pyrophosphate-exchange activity

Although isoleucine and leucine are, of the amino acids tested, the most effective in both incorporation and pyrophosphate exchange, no very clear correlation exists between pyrophosphate-exchange

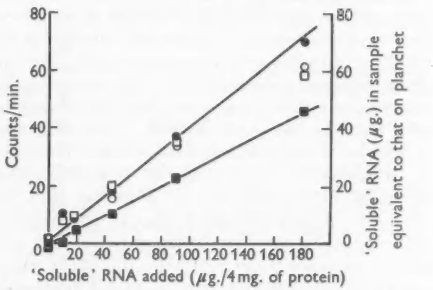

Fig. 6. Relationship between [^{14}C]amino acid incorporation into 'soluble' ribonucleic acid in a 'recombined' system and the amount of 'soluble' ribonucleic acid added. The assay system used is described under Materials and Methods. Each incubation mixture contained 4 mg. of pig-liver ASAc fraction and pig-liver 'soluble' RNA was added in the amounts indicated. Radioactivity is expressed as observed counts/min. (after correcting for self-absorption and the background counts of 10 counts/min.) in experiments with [^{14}C]leucine (●) and [^{14}C]valine (■). 'Soluble' RNA determinations were carried out upon portions identical with those used for the estimation of radioactivity, and the amounts of 'soluble' RNA found in these portions in the experiment with [^{14}C]leucine (○) and with [^{14}C]valine (□) are also shown. With 180 μg. of 'soluble' RNA added to the system the incorporation of leucine was equivalent to 1·8 μm-moles and of valine to 1·3 μm-moles/mg. of 'soluble' RNA. The standard errors for these points, calculated from the standard error of the ^{14}C counting, and reading from left to right, are: (i) leucine $\pm$4·1, $\pm$2·0, $\pm$2·5, $\pm$3·2, $\pm$4·3; (ii) valine 0, $\pm$1·9, $\pm$2·3, $\pm$2·5, $\pm$3·8.

activity and the ability of the preparations to incorporate [^{14}C]amino acids into 'soluble' ribonucleic acid. Incorporation of [^{14}C]amino acid into 'soluble' ribonucleic acid can occur in the absence of significant pyrophosphate-exchange activity. This may simply mean that the incorporation of [^{14}C]amino acids into 'soluble' ribonucleic acid is a more sensitive test for the presence of amino acid-activating enzymes than is pyrophosphate exchange, and that all the amino acid-activating enzymes of the liver are of the type described by Acs, Hartmann, Bowman & Lipmann (1959).

If we assume that the attachment of the amino acid to 'soluble' ribonucleic acid proceeds via an amino acyl adenylate, under our experimental conditions the rate and extent of the adenylate formation greatly exceeds that of the attachment of the amino acids to 'soluble' ribonucleic acid. This may in part reflect a lack of saturation with 'soluble' ribonucleic acid at the ribonucleic acid concentrations of the order used here, whereas the reactants in the pyrophosphate exchange are at near-saturation levels; but it may also reflect a difference in the maximum rates for the two reactions.

In our experiments with [^{14}C]valine we observed that pig-liver preparations are better able than rat-liver preparations to catalyse the transfer of this amino acid to 'soluble' ribonucleic acid. Pig-liver ASAc fraction is more effective in promoting the attachment of [^{14}C]valine to 'soluble' ribonucleic acid than is the rat-liver pH 5·0 fraction from which the ribonucleic acid was isolated. This effect is not related to the rates of valine-dependent exchange of pyrophosphate observed with the different preparations used, and is not observed with the more actively incorporated amino acids leucine and isoleucine. Perhaps the effect can be observed only when the conditions for the transfer of an amino acid to 'soluble' ribonucleic acid are in some way suboptimum.

Rendi & Campbell (1959) have shown that the protamine-treated 'S-protein' of Sachs (1957) will attach amino acids to added 'soluble' ribonucleic acid. We also observed that this preparation of Sachs can incorporate [^{14}C]amino acids into 'soluble' ribonucleic acid, but did not investigate this system as extensively as did Rendi & Campbell. Recently Rendi & Hultin (1959) have described a protamine-treated preparation similar to 'S-protein', which, like our ASAc fraction, is able to incorporate [^{14}C]leucine into added 'soluble' ribonucleic acid, but, unlike the ASAc fraction, cannot catalyse pyrophosphate exchange.

SUMMARY

1. Extracts of pig liver contain 50% of the soluble protein and 15–20% of the particulate material released by homogenizing the tissue by orthodox methods. The particulate material can be removed by treatment of the extract with protamine sulphate, followed by centrifuging at relatively low speeds.

2. Precipitation of the protamine-treated extract with ammonium sulphate and potassium acetate yields a protein fraction which will catalyse the activation of several amino acids, as measured by exchange of [^{32}P]pyrophosphate, to an extent very similar to that observed with pH 5·0 fractions prepared from the extract.

3. This protein fraction is very low in ribonucleic acid and is non-precipitable at pH 5·0 unless ribonucleic acid be added. The material with little ribonucleic acid readily catalyses the incorporation of [^{14}C]amino acids into added isolated 'soluble' ribonucleic acid obtained from either pig or rat liver.

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REFERENCES

Acs, G., Hartmann, G., Bowman, H. G. & Lipmann, F. (1959). *Fed. Proc.* **18**, 178.
Albaum, H. G. & Umbreit, W. W. (1947). *J. biol. Chem.* **167**, 369.
Allen, R. J. L. (1940). *Biochem. J.* **34**, 858.
Berg, P. (1956). *J. biol. Chem.* **222**, 1025.
Berg, P. & Ofengand, E. J. (1958). *Proc. nat. Acad. Sci., Wash.*, **44**, 78.
Crane, R. K. & Lipmann, F. (1953). *J. biol. Chem.* **201**, 235.
Davidson, J. N. & Waymouth, C. (1944). *Biochem. J.* **38**, 375.
Davie, E. W., Koningsberger, V. V. & Lipmann, F. (1956). *Arch. Biochem. Biophys.* **65**, 21.
Decken, von der, A. & Hultin, T. (1959). *Exp. Cell Res.* **17**, 188.
Finch, L. R. & Hele, P. (1959). *Biochem. J.* **73**, 11P.
Gornall, A. G., Bardawill, C. J. & David, M. M. (1949). *J. biol. Chem.* **177**, 751.
Grunert, R. R. & Phillips, P. H. (1951). *Arch. Biochem.* **30**, 217.
Hoagland, M. B. (1955). *Biochim. biophys. Acta*, **16**, 288.
Hoagland, M. B., Keller, E. B. & Zamecnik, P. C. (1956). *J. biol. Chem.* **218**, 345.
Hoagland, M. B., Stephenson, M. L., Scott, F. J., Hecht, L. I. & Zamecnik, P. C. (1958). *J. biol. Chem.* **231**, 241.
Hoagland, M. B., Zamecnik, P. C. & Stephenson, M. L. (1957). *Biochim. biophys. Acta*, **24**, 215.
Keller, E. B. & Zamecnik, P. C. (1956). *J. biol. Chem.* **221**, 45.
Kirby, K. S. (1956). *Biochem. J.* **64**, 405.
Lee Peng, C. H. (1956). *Biochim. biophys. Acta*, **22**, 42.
Lipmann, F. (1958). *Proc. nat. Acad. Sci., Wash.*, **44**, 67.
Novelli, G. D. (1958). *Proc. nat. Acad. Sci., Wash.*, **44**, 86.

Ogata, K. & Nohara, H. (1957*a*). *Biochim. biophys. Acta*, **25**, 659.
Ogata, K. & Nohara, H. (1957*b*). *Biochim. biophys. Acta*, **26**, 656.
Rendi, R. & Campbell, P. N. (1959). *Biochem. J.* **72**, 435.
Rendi, R., Di Milia, A. & Fronticelli, C. (1958). *Biochem. J.* **70**, 62.
Rendi, R. & Hultin, T. (1959). *Exp. Cell Res.* **17**, 540.
Sachs, H. (1957). *J. biol. Chem.* **228**, 23.
Schweet, R. S. & Allen, E. H. (1958). *J. biol. Chem.* **233**, 1104.
Schweet, R. S., Bovard, F. C., Allen, E. & Glassman, E. (1958). *Proc. nat. Acad. Sci., Wash.*, **44**, 173.
Simpkin, J. L. & Work, T. S. (1957). *Biochem. J.* **65**, 307.
Stadtman, E. R., Novelli, G. D. & Lipmann, F. (1951). *J. biol. Chem.* **191**, 365.
Webster, G. C. (1959). *Arch. Biochem. Biophys.* **82**, 125.
Zamecnik, P. C., Stephenson, M. L. & Hecht, L. I. (1958). *Proc. nat. Acad. Sci., Wash.*, **44**, 73.

Biochem. J. (1960) **75**, 363

Reactivation of Phosphorylated Acetocholinesterases by Pyridinium Aldoximes and Related Compounds

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Certain organophosphates, e.g. tetraethyl pyrophosphate, di*iso*propyl phosphorofluoridate and insecticides of the organophosphate type, inhibit acetocholinesterase by a phosphorylation process. The phosphorylated enzyme is devoid of esterase activity, but this can be restored by substances which displace the phosphoryl group from the enzyme. The best known reactivators are oximes and the most potent oxime is 2-hydroxyiminomethyl-*N*-methylpyridinium iodide (pyridine 2-aldoxime methiodide).

Because of the need for an antidote in organophosphate poisoning which is more effective and of greater water-solubility than 2-hydroxyiminomethyl-*N*-methylpyridinium iodide (Hobbiger & Sadler, 1959) we have investigated a series of pyridinium aldoximes and related compounds. In each case the reaction with tetraethyl pyrophosphate, the reactivation of diethyl phosphorylacetocholinesterase and the affinity for acetocholinesterase were investigated. In some instances the reaction with di*iso*propyl phosphorofluoridate and the reactivation of di*iso*propyl phosphorylacetocholinesterase were also studied.

It was found that some of the pyridinium aldoximes investigated are much more potent than 2-hydroxyiminomethyl-*N*-methylpyridinium iodide as reactivators of both diethyl and di*iso*propyl phosphorylacetocholinesterase and that their potency as reactivators cannot be predicted solely from their reactivity with organophosphates or from their affinity for acetocholinesterase, as measured by the anticholinesterase action.

EXPERIMENTAL

Methods

All experiments were carried out in the Warburg apparatus at 37° in a medium of 0·025 M-$NaHCO_3$ and an atmosphere of $N_2 + CO_2$ (95:5), pH 7·45. All vessels were gassed while being shaken with the mixture of $N_2 + CO_2$ for 5 min. after their transfer to the water bath. All incubation periods mentioned in the text include this period.

Reactivity of oximes with organophosphates. The reaction between oximes and organophosphates leads to the release of acids; this reaction was followed by measuring the displacement of CO_2 from the $NaHCO_3$ medium and its rate constant k, expressed as $l.mole^{-1}min.^{-1}$, was calculated from the formula

$$k = \frac{2{\cdot}303}{[\text{Reactivator}]\,(t_2 - t_1)} \log \frac{a}{a-b}.$$

2 mM-Tetraethyl pyrophosphate or di*iso*propyl phosphorofluoridate was used in these experiments; a represents $\mu l.$ of CO_2 obtained when 2 moles of acid/mole of organophosphate are released. Ten minutes were allowed for equilibration in the water bath and t_1 is the time of addition of the organophosphate from the side arm to the oxime in the main compartment. Only measurements of initial rates were taken and $t_2 - t_1$ are 10 and 20 min. in experiments with tetraethyl pyrophosphate and di*iso*propyl phosphorofluoridate respectively; b is $\mu l.$ of CO_2 produced during this period. Whenever possible concentrations of each oxime were used which on interaction with the organophosphate produced a CO_2 output differing by not more than 10% from that obtained with one of two standard concentrations of 2-hydroxyiminomethyl-*N*-methylpyridinium iodide. In all experiments spontaneous hydrolysis of the organophosphate contributed at the most 20% to the observed hydrolysis and no correction was made for this when calculating rate constants.

Reactivation of phosphorylated acetocholinesterases. Washed human erythrocytes or purified bovine-erythrocyte cholinesterase (Winthrop-Stearns Inc.) were used as enzyme source and acetocholinesterase activity was determined by the rate of hydrolysis of 0·01 M-acetylcholine chloride. Final concentrations for the assay of activity of the two enzyme preparations, which are referred to below as human and bovine acetocholinesterase, were 1·7 ml. of washed human erythrocytes/100 ml. and 40 μg. of purified enzyme/ml. respectively in a volume of 3 ml. per Warburg vessel.

Preparations of human diethyl and di*iso*propyl phosphorylacetocholinesterase were obtained by a technique described previously (Hobbiger, 1957). The corresponding phosphorylated bovine acetocholinesterases were prepared in the same way, except that after incubation of the enzyme with the organophosphate and removal of excess of the latter by incubation with rabbit plasma a tenfold dilution was substituted for the procedure of multiple washings.

To assess their relative potencies as reactivators the oximes were incubated with the phosphorylated enzyme in the main compartment of the manometric vessels for 30 min. before addition of substrate; the degree of reactivation obtained was calculated from the CO_2 production during 5–35 min. after the addition of substrate from the expression: Percentage reactivation $= 100\,(A - B)/(C - B)$, where A is the activity of reactivated enzyme, B the activity of phosphorylated enzyme and C the activity obtained by maximum reactivation of phosphorylated enzyme. Under these conditions $(A - B)$ is the sum of (i) the reactivation which occurs before addition of substrate, (ii) the reactivation which takes place from the time of addition of substrate until 35 min. later. Since the latter was found to have a first-order dependence on the concentration of phosphorylated enzyme the calculated percentage reactivation represents the reactivation which was obtained at 20 min. after the addition of substrate. For this reason certain lines in Figs. 1 and 2 do not pass through the origin.

Relative potencies were calculated from the concentrations of reactivators which gave similar percentage reactivations. For studies of the time course of reactivation the same procedure as that employed for assessment of the relative potencies of reactivators was used except that the period of incubation without substrate was varied.

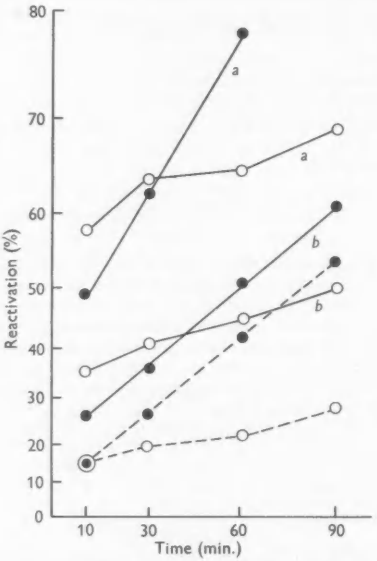


Fig. 1. Reactivation of human diethyl phosphorylacetocholinesterase obtained when 2-hydroxyiminomethyl-*N*-methylpyridinium iodide (●—●; *a* 0·012 mM, *b* 0·006 mM) or *NN'*-trimethylenebis-(4-hydroxyiminomethylpyridinium bromide) (○—○; *a* 0·0005 mM, *b* 0·00025 mM) was incubated with the phosphorylated enzyme in the main compartment of the manometric vessel for various periods (abscissa) before addition of substrate. Under these conditions reactivation takes place during measurement of enzyme activity for assessment of reactivation. Broken lines represent reactivation when 0·006 mM-2-hydroxyiminomethyl-*N*-methylpyridinium iodide (●– – –●) and 0·00025 mM-*NN'*-trimethylenebis-(4-hydroxyiminomethylpyridinium bromide) (○– – –○) were incubated for various periods (abscissa) in the side arm of the manometric vessels before transfer to the substrate in the main compartment. Only negligible reactivation takes place during measurement of enzyme activity.

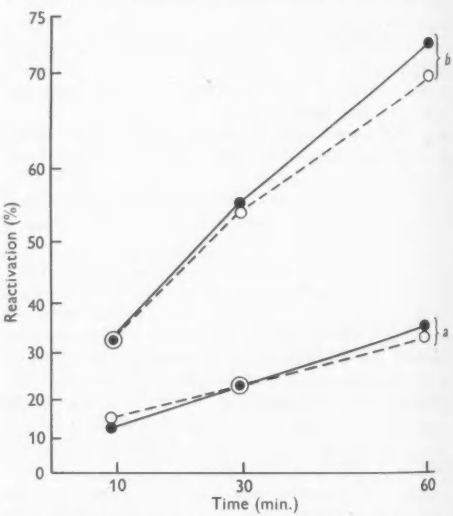


Fig. 2. Reactivation of bovine diethyl phosphorylacetocholinesterase obtained when 2-hydroxyiminomethyl-*N*-methylpyridinium iodide (●; *a* 0·006 mM; *b* 0·018 mM) or *NN'*-trimethylenebis-(4-hydroxyiminomethylpyridinium bromide) (○; *a* 0·0002 mM; *b* 0·0006 mM) were incubated for various periods (abscissa) with the phosphorylated enzyme in the main compartment of the manometric vessel before addition of substrate from the side arm.

Anticholinesterase activity. Fresh human acetocholinesterase and oximes were incubated in the main compartment for 20 min. and I_{50} values were calculated from enzyme activities recorded between 5 and 35 min. after addition of substrate from the side arm.

Relative potencies. The highest concentration of oximes used in any experiment was 0·01 M and all results were expressed relative to those obtained with 2-hydroxyiminomethyl-*N*-methylpyridinium iodide (activity of 1). The lowest relative potencies which could be recorded with accuracy were 0·05 for anticholinesterase activity and reactivity with organophosphates and 0·0003 and 0·005 for reactivation of human diethyl and di*iso*propyl phosphorylacetocholinesterase respectively.

The pK_a values listed are the pH values at the half-titration point of 0·03 m-moles in 5 ml. of water at room temperature. A continuous titrator (titrigraph), model SBR 2a, was used for all titrations.

Reactivators

Oximes and related substances were all synthesized in this Laboratory. The method of synthesis, analytical data and solubilities in chloroform and water of oximes listed in Table 1 have been reported in a previous publication (Hobbiger & Sadler, 1959). Oximes listed in Table 3 and 2-hydroxyiminomethyl-*N*-*iso*propylpyridinium iodide were synthesized by the following methods.

β-Pyridinium acetoxime chloride. Excess of pyridine was added to a solution of β-chloroacetoxime in ethanol, and allowed to stand for several hours. The excess of pyridine and ethanol was removed by distillation *in vacuo*. The residue was triturated with acetone and then crystallized from ethanol–acetone, giving white plates, m.p. 193°. (Found: Cl, 20·5. Calc.: Cl, 20·6%).

Betaine aldoxime chloride. This was prepared similarly from trimethylamine and β-chloroacetoxime in ethanol. White plates, m.p. 81°, were obtained with 1 mol. of water of crystallization from aqueous acetone (Found: C, 35·4; H, 8·2; N, 17·1; Cl, 21·2. Calc.: C, 35·3; H, 8·8; N, 16·5; Cl, 20·9%).

2-Hydroxyiminomethylpyridinium acetoxime chloride. This was prepared as described above from 2-hydroxyiminomethylpyridine and β-chloroacetoxime in ethanol. Crystallization from ethanol–acetone afforded cream needles, m.p. 196–197° (Found: Cl, 16·6. Calc.: Cl, 16·5%).

4-Hydroxyiminomethyl-N-methylquinolinium methiodide. This was obtained as orange needles from ethanol, m.p. 242° (Ginsburg & Wilson, 1957).

4-Hydroxyiminomethyl-6-methoxy-N-methylquinolinium iodide. This was prepared as described above from 4-formyl-6-methoxyquinoline; orange needles from methanol, m.p. 225°, were obtained (Found: I, 36·8. Calc.: I, 36·9%).

2-Hydroxyiminomethyl-N-isopropylpyridinium iodide. Equimolar quantities of 2-hydroxyiminomethylpyridine and *iso*propyl iodide in ethanol were heated under reflux for 8 hr. The solid obtained on the addition of ether to the cooled reaction mixture gave lemon-yellow needles, m.p. 208° after crystallization from methanol (Found: I, 43·7. Calc.: I, 43·5%).

2-Hydroxyiminomethyl-3:3-dimethyl-N-methylindoleninium iodide. Reaction of nitrous acid with 2:3:3-trimethylindolenine (obtained by a Fischer indole synthesis) gave 2-hydroxyiminomethyl-3:3-dimethylindolenine m.p. 156° (Found: C, 70·0; H, 6·4. Calc. C, 70·2; H, 6·4%). The quaternary compound was prepared by heating the product with excess of methyl iodide in nitrobenzene. The product which separated on cooling afforded deep-yellow plates from methanol, m.p. 192° (Found: C, 43·7; H, 4·8; N, 8·5; I, 38·2. Calc.: C, 43·6; H, 4·5; N, 8·5; I, 38·5%).

RESULTS

Hydrolysis of organophosphates

Hydrolysis of tetraethyl pyrophosphate. The hydrolysis of tetraethyl pyrophosphate by 19 pyridinium aldoximes was investigated at pH 7·45 and 37° (see Methods section) and the results obtained are summarized in Table 1. As can be seen the reactivity of hydroxyiminomethyl-*N*-alkylpyridinium iodides with tetraethyl pyrophosphate was greatly affected by the position of the oxime group relative to the ring nitrogen, but was independent of the size of the *N*-alkyl group. This also applied to derivatives with a terminal bromo substituent in the *N*-alkyl group.

4-Monoximes of *NN′*-polymethylenebis(pyridinium bromide) were 1·5–1·8 times as reactive as their parent compound 4-hydroxyiminomethyl-*N*-methylpyridinium iodide and 4:4′-dioximes had approximately twice the reactivity of their corresponding monoximes. In the dioximes with a polymethylene chain of 1–3 carbon atoms an inverse relationship existed between reactivity and length of the *NN′*-polymethylene group.

The amount of the oxime determined the extent to which tetraethyl pyrophosphate was hydrolysed if the latter was used in excess. This indicates that the oximes undergo a chemical change during hydrolysis. In agreement with this is the finding that mixtures of tetraethyl pyrophosphate and oximes when kept at 37° in 0·1 M-phosphate buffer, pH 7·4, gave a positive cyanide reaction, the intensity of which increased during incubation. Control solutions containing only tetraethyl pyrophosphate or oximes failed to give such a positive cyanide reaction.

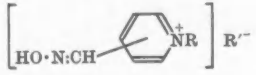
Hydrolysis of diisopropyl phosphorofluoridate. Differences in reactivity between the three isomeric hydroxyiminomethyl-*N*-methylpyridinium iodides were less marked when di*iso*propyl phosphorofluoridate was used instead of tetraethyl pyrophosphate (compare Tables 1 and 2). On the other hand, the reactivities of 4-monoximes and 4:4′-dioximes of *NN′*-polymethylenebis(pyridinium bromide) relative to 4-hydroxyiminomethyl-*N*-methylpyridinium iodide were very similar for both organophosphates.

Reactivation of freshly phosphorylated acetocholinesterases

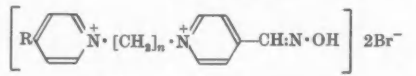
Human diethyl phosphorylacetocholinesterase. Amongst the three isomeric hydroxyiminomethyl-*N*-methylpyridinium iodides the 2-isomer was the

Table 1. *Biochemical properties of pyridinium and* bis*pyridinium aldoximes*

Potencies of individual oximes are expressed relative to that of 2-hydroxyiminomethyl-*N*-methylpyridinium iodide (potency of 1) and each figure was derived from the mean of several experiments, each of which was a parallel assay of the activities of 2-hydroxyiminomethyl-*N*-methylpyridinium iodide and several other oximes. 2-Hydroxyiminomethyl-*N*-methylpyridinium iodide had under the experimental conditions described in the Methods section the following potencies: (1) Reactivity: k 29·3 and 26·6 l. mole^{-1} min.$^{-1}$ for the hydrolysis of 2 mM-tetraethyl pyrophosphate by 1 and 2 mM-oxime respectively. (2) 50% reactivation of human and bovine diethyl phosphorylacetocholinesterases were obtained when the phosphorylated enzymes were incubated with 0·012 mM-oxime for 20 and 58 min. respectively before addition of substrate. (3) The oxime concentration required for 50% inhibition of acetylcholine hydrolysis was 4·2 mM.



Position of hydroxyiminomethyl group	R	R′	pK_a	Reactivity with tetraethyl pyrophosphate	Potency as reactivator of diethyl phosphorylacetocholinesterase: Human	Potency as reactivator of diethyl phosphorylacetocholinesterase: Bovine	Potency as inhibitor of acetylcholine hydrolysis by human acetocholinesterase
2	CH_3	I	8·1	1	1	1	1
3	CH_3	I	9·2	0·09	0·0003	—	1·2
4	CH_3	I	8·3	0·36	0·06	—	0·75
2	$CH_2 \cdot CH_3$	I	8·2	1	0·54	—	2·1
2	$[CH_2]_2 \cdot CH_3$	I	8·3	0·94	0·63	—	5·6
2	$[CH_2]_3 \cdot CH_3$	I	8·3	1·10	0·87	—	64
2	$CH(CH_3)_2$	I	8·0	0·95	0·46	—	1·6
4	$CH_2 \cdot CH_3$	I	8·2	0·34	0·03	—	0·53
4	$[CH_2]_2 \cdot CH_2 \cdot Br$	Br	7·9	0·44	1·9	—	17
4	$[CH_2]_3 \cdot CH_2 \cdot Br$	Br	8·2	0·36	0·25	—	22
4	$[CH_2]_4 \cdot CH_2 \cdot Br$	Br	8·2	0·27	0·94	—	6



R	n					
H	3	8·0	0·56	8	5	0·75
H	4	8·0	0·53	6·7	4·2	0·40
H	5	8·2	0·47	7·5	4·4	2·7
CH:N·OH	1	7·4	2·60	2·8	—	<0·1
CH:N·OH	2	8·0	1·50	17	20	0·78
CH:N·OH	3	8·0	1·10	22	30	0·90
CH:N·OH	4	7·9	0·99	18	16	1·0
CH:N·OH	5	8·2	0·91	16	13	8·1

most potent reactivator and the 4- and 3-isomers had only 6·0 and 0·03% respectively of the activity of the former. Enlargement of the *N*-alkyl group caused a slight loss of activity that was most marked with the ethyl and *iso*propyl derivatives, which had approximately 50% of the activity of the corresponding methyl derivatives.

The introduction of a terminal bromine atom into the *N*-alkyl group markedly enhanced the activity of 4-hydroxyiminomethyl-*N*-alkylpyridinium halides. The most potent reactivators were the 4-monoximes and 4:4′-dioximes of *NN′*-polymethylenebis(pyridinium bromide), which were 2·8–22 times as potent as reactivators as was 2-hydroxyiminomethyl-*N*-methylpyridinium iodide. In the largest homologous series available, namely the 4:4′-dioximes of *NN′*-polymethylenebis(pyridinium bromide), a marked increase in potency resulted from lengthening the *NN′*-polymethylene chain from 1 to 3 carbon atoms but activity then fell gradually when the polymethylene chain was lengthened still further.

These results are summarized in Table 1.

Factors influencing reactivation. The rate of reactivation of human diethyl phosphorylacetocholinesterase by 2-hydroxyiminomethyl-*N*-methylpyridinium iodide in the presence or absence of 0·01 mM-acetylcholine is a function of the oxime concentration, and with a fixed concentration of the oxime shows a first-order dependence on the concentration of phosphorylated enzyme (Hobbiger 1956). This also applied to reactivation by 4-hydroxyimino-*N*-methylpyridinium iodide. When oximes of *NN′*-polymethylenebis(pyridinium bromide) were used as reactivators, however, the observed time course of reactivation was decremental with respect to that obtained with 2-hydroxyiminomethyl-*N*-methylpyridinium iodide. The decrement was more marked with the 4:4′-dioximes than with the 4-monoximes, unaffected by 0·1–1·0 mM-disodium ethylenediaminetetra-acetate, and of a similar order when different oximes of a given group were compared. The time courses of reactivation obtained with concentrations of *NN′*-trimethylenebis-(4-hydroxyiminomethylpyridinium bromide) and 2-hydroxyiminomethyl-*N*-methylpyridinium iodide, which produced similar degrees of reactivation when the oximes were added to the phosphorylated enzyme 30 min. before acetylcholine, are illustrated in Fig. 1.

When substrate was added simultaneously with oximes of *NN′*-polymethylenebis(pyridinium bromide) to the phosphorylated enzyme instead of at a later stage (as was the case when the time course of reactivation was studied) the rate of reactivation showed a first-order dependence on the concentration of phosphorylated enzyme. This was also true for the reactivation which occurred after addition of substrate to a sample containing phosphorylated enzyme exposed previously for some time to any of the oximes.

Thus the cause of the observed decremental time course of reactivation by oximes of *NN′*-polymethylenebis(pyridinium bromide) lies in an interference with reactivation which occurs during the period while the phosphorylated enzyme is incubated with the oxime in the absence of substrate and which is prevented by substrate. Since it is known that acetylcholine protects acetocholinesterase against phosphorylation (Burgen, 1949) and free organophosphates can interfere with reactivation (Hobbiger, 1955, 1956), it was necessary to show that the observed interference was not due to free tetraethyl pyrophosphate.

For this purpose preparations of human diethyl phosphorylacetocholinesterase were incubated for 60 min. with fresh human acetocholinesterase as control enzyme. Although the former was used in a concentration which was 10 times as high as that used in reactivation studies no significant inhibition of the control enzyme could be demonstrated. Similarly a tenfold increase in the concentration of tetraethyl pyrophosphate during preparation of the phosphorylated enzyme did not affect results significantly. Thus it seems unlikely that residual free tetraethyl pyrophosphate could to any significant extent have been responsible for the anomalous time course of reactivation obtained with oximes of *NN′*-polymethylenebis(pyridinium bromide) in the absence of substrate.

Differences between the time courses of reactivation obtained with 2-hydroxyiminomethyl-*N*-methylpyridinium iodide and oximes of *NN′*-polymethylenebis(pyridinium bromide) were pH-dependent and became negligible when reactivation was carried out at pH 8·45 instead of at pH 7·45.

The relative potencies of oximes of *NN′*-polymethylenebis(pyridinium bromide) calculated from results obtained in experiments in which oximes and substrate were added simultaneously to the phosphorylated enzyme were 1·2–1·5 times as great as those obtained when the substrate was added to the phosphorylated enzyme 30 min. after the oxime.

Bovine diethyl phosphorylacetocholinesterase. Oximes of *NN′*-polymethylenebis(pyridinium bromide) are more potent than 2-hydroxyiminomethyl-*N*-methylpyridinium iodide as reactivators not only of diethyl phosphorylacetocholinesterase of lysed human erythrocytes but also of the diethyl phosphoryl derivative of purified bovine-erythrocyte cholinesterase. The similarity between results obtained under identical experimental conditions with the two types of diethyl phosphorylacetocholinesterase can be seen from the results presented in Table 1.

Differences between time courses of reactivation of bovine diethyl phosphorylacetocholinesterase obtained with oximes of *NN'*-polymethylenebis-(pyridinium bromide) and 2-hydroxyiminomethyl-*N*-methylpyridinium iodide were, on the whole, quite small (see Fig. 2).

Human di-isopropyl phosphorylacetocholinesterase. Oximes of *NN'*-polymethylenebis(pyridinium bromide) were also potent reactivators of human di-*iso*-propyl phosphorylacetocholinesterase when incubated with the phosphorylated enzyme for 30 min. before addition of substrate (Table 2). *NN'*-Trimethylenebis-(4-hydroxyiminomethylpyridinium bromide) was the most potent reactivator, being 52 times as effective as 2-hydroxyiminomethyl-*N*-methylpyridinium iodide. Differences in potencies between the oximes of *NN'*-polymethylenebis-(pyridinium bromide) were in line with those encountered in experiments with human diethyl phosphorylacetocholinesterase. Differences between the time courses of reactivation obtained with oximes of *NN'*-polymethylenebis(pyridinium bromide) and 2-hydroxyiminomethyl-*N*-methylpyridinium iodide were small and of a similar order to those observed in experiments with bovine diethyl phosphorylacetocholinesterase.

Reactivation of 'aged' phosphorylated acetocholinesterase

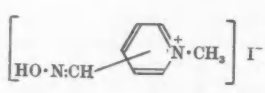
The maximum degree of reactivation of phosphorylated human cholinesterases obtainable with 2-hydroxyiminomethyl-*N*-methylpyridinium iodide or related compounds is inversely related to the 'age' of the phosphorylated enzyme (Hobbiger, 1955, 1956; Davies & Green, 1956). It was found that this was also the case when oximes of *NN'*-polymethylenebis(pyridinium bromide) were used as reactivators of human di-*iso*propyl phosphorylacetocholinesterase. Similar results were obtained by Michel (1958), who studied the effect of 'age' on reactivation of methyl-*iso*propyl phosphorylacetocholinesterase by *NN'*-tetramethylenebis-(4-hydroxyiminomethylpyridinium bromide).

Inhibition of human acetocholinesterase by oximes

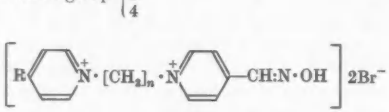
The anticholinesterase action of oximes was assessed by their effect on the rate of hydrolysis of acetylcholine by human acetocholinesterase. Differences in inhibitory potency between the three isomeric hydroxyiminomethyl-*N*-methylpyridinium iodides were small, and Table 1 shows that the anticholinesterase activities of 2-hydroxyiminomethyl-

Table 2. *Biochemical properties of pyridinium and* bis*pyridinium aldoximes*

All results are expressed relative to those obtained with 2-hydroxyiminomethyl-*N*-methylpyridinium iodide (see legend of Table 1). 2-Hydroxyiminomethyl-*N*-methylpyridinium iodide had under the experimental conditions described in the Methods section the following potencies: (1) reactivity with di-*iso*propyl phosphorofluoridate: k 3·56 l. mole^{-1} min.$^{-1}$ for the hydrolysis of 2 mM-di-*iso*propyl phosphorofluoridate by 2 mM-oxime. (2) 24 min. incubation of human di-*iso*propyl phosphorylacetocholinesterase with 0·15 mM solutions of the oxime before addition of substrate was required to give 50% reactivation.



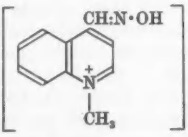
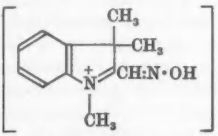
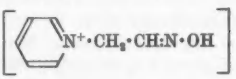
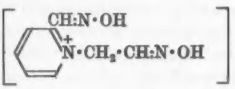
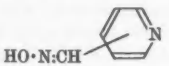
position of oxime group	Reactivity with di-*iso*propyl phosphorofluoridate	Potency as reactivator of human di-*iso*propyl phosphorylacetocholinesterase
2	1	1
3	0·2	—
4	0·65	—



R	n		
H	3	1	11
H	4	0·94	10
H	5	0·92	11
CH:N·OH	1	3·5	—
CH:N·OH	2	3·1	22
CH:N·OH	3	2	52
CH:N·OH	4	1·9	38
CH:N·OH	5	1·8	36

Table 3. *Biochemical properties of related oximes*

Potencies are expressed relative to those of 2-hydroxyiminomethyl-*N*-methylpyridinium iodide (potency of 1). For further details see legend to Table 1.

	pK_a	Reactivity with tetraethyl pyrophosphate	Potency as reactivator of human diethyl phosphorylacetocholinesterase	Potency as inhibitor of acetylcholine hydrolysis by human acetocholinesterase
	8·2	0·78	0·035	10
its 6-methoxy derivative	8·4	0·37	0·008	14
	5·8	0·63	0·004	<0·1
$[(CH_3)_3\overset{+}{N}\cdot CH_2\cdot CH{:}N\cdot OH]\ Cl^-$	9·0	0·05	0·0005	<0·1
	—	0·04	<0·0004	<0·1
	7·9	0·83	0·2	2·8
	—	<0·02	<0·0004	<0·1

N-alkylpyridinium iodides, ω-bromo derivatives of 4-hydroxyiminomethyl-*N*-alkylpyridinium bromides and oximes of *NN'*-polymethylenebis(pyridinium bromide) were, on a whole, directly related to the size of the *N*-alkyl group and the length of the *NN'*-polymethylene chain. 4-Hydroxyiminomethyl-*N*-ethylpyridinium iodide, the ω-bromo derivative of 4-hydroxyiminomethyl-*N*-amylpyridinium bromide and *NN'*-tetramethylene-1-(4-hydroxyiminomethylpyridinium)-4-pyridinium dibromide were the only exceptions.

Experiments with reactivators closely related to pyridinium aldoximes

Additional experiments in which the effects of reactivators, listed in Table 3, on tetraethyl pyrophosphate, human diethyl phosphorylacetocholinesterase and human acetocholinesterase were investigated gave the following results.

4-Hydroxyiminomethyl-*N*-methylquinolinium iodide, its 6-methoxy derivative and 2-hydroxyiminomethyl-3:3-dimethyl-*N*-methylindoleninium iodide had 78, 37 and 63% respectively of the reactivity of 2-hydroxyiminomethyl-*N*-methylpyridinium iodide. The first two oximes were much more potent than 2-hydroxyiminomethyl-*N*-methylpyridinium iodide as inhibitors of acetocholinesterase, but their potencies as reactivators relative to 2-hydroxyiminomethyl-*N*-methylpyridinium iodide were only 3·5 and 0·8% respectively. The indoleninium derivative also was only a very weak reactivator.

β-Pyridinium acetoxime chloride and betaine aldoxime chloride, which, like 3-hydroxyiminomethyl-*N*-methylpyridinium iodide, lack a fully resonating structure but have (as far as complementariness for the phosphorylated enzyme is concerned) the same structural configuration as 2-hydroxyiminomethyl-*N*-methylpyridinium iodide, had very low reactivities and their potencies as reactivators were too small to be recorded with accuracy. The same seems to be true for β-dimethylanilinium acetoxime chloride, although it was not possible to get accurate results

with this compound because of its ready decomposition under the experimental conditions.

Replacement of the *N*-methyl group of 2-hydroxyiminomethyl-*N*-methylpyridinium iodide by $N\text{-}CH_2\cdot CH{:}N\cdot OH$ yielded an oxime with lower reactivity and lower potency as reactivator than 2-hydroxyiminomethyl-*N*-methylpyridinium iodide.

Only trace activities were observed with the three isomers of hydroxyiminomethylpyridine when they were used in 0·01 M concentrations.

These results are summarized in Table 3.

2-Hydroxyiminomethylthiophen, gramine methiodide, Girard's P and T reagents (Girard & Sandulesco, 1936), *p*-nitrosodimethylaniline and *p*-hydroxyiminomethyltrimethylanilinium bromide were devoid of action on both tetraethyl pyrophosphate and human diethyl phosphorylacetocholinesterase when used in 0·01 M concentrations. Similarly ineffective were saturated solutions of the following compounds: 2- and 4-hydroxyiminomethylquinoline and the 6-methoxy derivative of the latter, 4-carboxyl-2-methylquinoline *N*-oxide, 2-methylcinchoninic acid hydrazide and its 6-fluoro derivative, 2-hydroxyiminomethyl-3:3-dimethylindolenine, 3-hydroxyiminomethylindole, 4:4′-di*iso*nitrosoacetaminodiphenyl sulphone, 2:6-dihydroxyiminomethylpyridine, 1-methyl-3-hydroxyiminomethyloxindole and a large series of halogen-, alkyl- and alkoxy-substituted isatin *β*-oximes and their corresponding *iso*nitrosoacetanilides.

DISCUSSION

Results presented in this paper show that the activity of diethyl and di*iso*propyl phosphorylacetocholinesterase can be restored by a variety of pyridinium aldoximes and bispyridinium aldoximes. Wilson, Ginsburg & Quan (1958), who carried out an extensive study of 2-hydroxyiminomethyl-*N*-methylpyridinium iodide and of related compounds, came to the conclusion that the exceptional activity of the former as a reactivator of diethyl phosphorylacetocholinesterase is a consequence of its very high degree of complementariness with phosphorylated acetocholinesterase. They envisage that bonding of the quaternary ammonium group of this oxime to the anionic site of the acetocholinesterase enhances the activity of the oxime as a reactivator by facilitating alignment between the oxime and phosphoryl groups. Furthermore, they believe that the same type of complementariness is responsible for the high activity of 4-hydroxyiminomethyl-*N*-methylpyridinium iodide, whereas the 3-isomer, which is stated to have less than a millionth of the activity of the 2-isomer, is inactive because it lacks complementariness. According to our results the 3-isomer still has 0·5 % of the activity of the 2-isomer as a reactivator if allowance is made for differences in reactivity of the two oximes with organophosphates.

To assess the extent to which the activities of isomeric hydroxyiminomethyl-*N*-methylpyridinium iodides as reactivators also reflected mesomeric properties, we investigated betaine aldoxime chloride and *β*-pyridinium acetoxime chloride, which structurally resemble 2-hydroxyiminomethyl *N*-methylpyridinium iodide but lack a fully conjugated structure. Both these oximes have reactivities with organophosphates similar to that of 3-hydroxyiminomethyl-*N*-methylpyridinium iodide and share its low activity as a reactivator. Unfortunately their affinity for acetocholinesterase, as measured by the anticholinesterase activity, is less than 10 % of that of 3-hydroxyiminomethyl-*N*-methylpyridinium iodide. Thus no definite conclusion about the contribution towards reactivation by mesomeric and inductive effects can be drawn from our experiments. However, all known potent reactivators possess a conjugated structure.

The effectiveness of 2- and 4-hydroxyiminomethyl-*N*-alkylpyridinium iodides as reactivators can be explained satisfactorily by their complementariness, as proposed by Wilson *et al.* (1958). Although the affinity of these oximes for acetocholinesterase increases with increasing length of *N*-alkyl group, spatial limitations at the anionic site of the phosphorylated enzyme (Wilson, 1955) would tend to reduce their affinity for the anionic site of the phosphorylated enzyme and this could account for the superiority of the methyl derivatives as reactivators. The low activity of 4-hydroxyiminomethyl-*N*-methylquinolinium iodide, its 6-methoxy derivative and 2-hydroxyiminomethyl-3:3-dimethyl-*N*-methylindoleninium iodide as reactivators requires the postulate that spatial limitations at the anionic site are considerable. In support of this interpretation is the low affinity of the indoleninium derivative for acetocholinesterase.

The *ω*-bromo derivatives of 4-hydroxyiminomethyl-*N*-alkylpyridinium bromides are 4–25 times as effective as reactivators as their parent compound 4-hydroxyiminomethyl-*N*-methylpyridinium iodide, as judged by the ratio (potency as reactivator : reactivity with organophosphates). If we assume that the quaternary-ammonium group of these oximes is bonded to the anionic site of the phosphorylated enzyme, their superiority to 4-hydroxyiminomethyl-*N*-methylpyridinium iodide could be accomplished only by a bonding of the terminal *ω*-bromo group to a second site on the enzyme surface. Such additional bonding would then be able to exert a directive influence for the orientation of the oxime. In view of the spatial restrictions of the anionic site outlined above the

second bonding site could not be part of the anionic site but would have to be adjacent to it.

The oximes of *NN'*-polymethylenebis(pyridinium bromide) are, according to the results summarized in Tables 1 and 2, at least 6·5–120 and 1·1–26 times as effective as reactivators as are 4-hydroxyiminomethyl-*N*-methylpyridinium iodide and its 2-isomer respectively, if allowance is made for differences in reactivity with organophosphates. Corresponding pairs of monoximes and dioximes have ratios (potency as reactivator:reactivity with organophosphates) which differ by a factor of approximately 2. Again there is no correlation between anticholinesterase activity and activity as a reactivator. To explain the high activity of oximes of *NN'*-polymethylenebis(pyridinium bromide) as reactivators we should have to postulate bonding of the quaternary-ammonium groups to two sites of the enzyme surface, and the finding that the trimethylene derivative has the highest activity then indicates that these two points of attachment are separated by a distance not exceeding 5 Å. The finding of Berry, Davies & Green (1959) that *NN'*-trimethylenebis(2-hydroxyiminomethylpyridinium bromide) is a less potent reactivator than its hexamethylene-derivative does not contradict this, because of the steric differences between these oximes and those which we have investigated.

Our findings are also in agreement with the interpretation that both oxime groups are functional during reactivation, and the possibility must therefore be considered that pyridinium aldoximes with a single quaternary-ammonium group might be bonded to a site which is not identical with the anionic site but which represents the second bonding site discussed above. Nothing is known about the nature of such a second site for attachment of quaternary-ammonium groups of pyridinium aldoximes. However, strong bonding between imidazoles and quaternary-pyridinium derivatives has been demonstrated by van Eys (1958). Receptors for acetylcholine, which must share at least certain features with the active surface of acetocholinesterase, are most readily stimulated by alkylammonium derivatives which have a small cationic head and a second nucleophilic group, e.g. a sulphur atom, between the third and fourth carbon atom of the appendix structure (Lands & Cavalitto, 1954).

Reactivation of human diethyl phosphorylacetocholinesterase (of lysed human erythrocytes) by oximes of *NN'*-polymethylenebis(pyridinium bromide) in the absence of substrate deviates markedly from that obtained with 2-hydroxyiminomethyl-*N*-methylpyridinium iodide. This deviation is much less in experiments with human di*iso*propyl phosphorylacetocholinesterase and bovine diethyl phosphorylacetocholinesterase. Several likely sources of interference with reactivation could be disproved. Since Somers & Bay (1959) have shown that an oxime phosphonate derived from 4-hydroxyiminomethyl-*N*-methylpyridinium iodide phosphorylates acetocholinesterase, it is possible that an oxime phosphonate is responsible for at least part of the interference with reactivation observed in our experiments. That interference with reactivation of human diethyl phosphorylacetocholinesterase was far greater than with bovine diethyl phosphorylacetocholinesterase does not necessarily contradict this interpretation, since the former preparation was a crude enzyme preparation whereas the latter represented a highly purified enzyme preparation. The interference with reactivation does not seem to be of practical importance, since oximes of *NN'*-polymethylenebis(pyridinium bromide) have a short duration of action *in vivo* (Hobbiger & Sadler, 1959) and are greatly superior to 2-hydroxyiminomethyl-*N*-methylpyridinium iodide both as reactivators (Hobbiger & Sadler, 1959) and as antidotes to poisoning by a variety of organophosphates (Bay, Krop & Yates, 1958; Hobbiger, O'Sullivan & Sadler, 1958; Hobbiger & Sadler, 1958, 1959; Berry *et al.* 1959).

The finding that the hydrolysis of organophosphates by pyridinium aldoximes and bispyridinium aldoximes yields cyanide needs further investigation in view of its possible clinical importance. The production of cyanide from aliphatic 2-oxo-oximes during their metabolic degradation *in vivo* has already been demonstrated by Askew, Davies, Green & Holmes (1956).

SUMMARY

1. The reactivation of diethyl and di*iso*propyl phosphorylacetocholinesterases by aldoximes of pyridinium and bispyridinium and related compounds was investigated. The anticholinesterase activity of these oximes and the hydrolysis of tetraethyl pyrophosphate and di*iso*propyl phosphorofluoridate by them were also studied. All experiments were carried out at pH 7·45 and 37°.

2. The most potent reactivators among the oximes studied were 4-monoximes and 4:4'-dioximes of *NN'*-polymethylenebis(pyridinium bromide), which were greatly superior as reactivators to 2-hydroxyiminomethyl-*N*-methylpyridinium iodide (pyridine 2-aldoxime methiodide). Reactivation of human diethyl phosphorylacetocholinesterase by oximes of *NN'*-polymethylenebis(pyridinium bromide) was faster in the presence of acetylcholine than without it. The hypothesis is put forward that an oxime phosphonate might be formed during reactivation and interfere with reactivation in the absence of acetylcholine.

3. There is no correlation between anticholinesterase activity and effectiveness as reactivator. The ratios (potency as reactivator:reactivity with organophosphates), obtained with different oximes, support the interpretation that attachment to the phosphorylated enzyme during reactivation enhances activity. Possible sites of attachment are discussed.

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REFERENCES

Askew, B. M., Davies, D. R., Green, A. L. & Holmes, R. (1956). *Brit. J. Pharmacol.* **11**, 424.
Bay, E., Krop, S. & Yates, L. F. (1958). *Proc. Soc. exp. Biol., N.Y.*, **98**, 107.
Berry, W. K., Davies, D. R. & Green, A. L. (1959). *Brit. J. Pharmacol.* **14**, 186.
Burgen, A. S. V. (1949). *Brit. J. Pharmacol.* **4**, 219.
Davies, D. R. & Green, A. L. (1956). *Biochem. J.* **63**, 529.
Ginsburg, S. & Wilson, I. B. (1957). *J. Amer. chem. Soc.* **79**, 481.
Girard, A. & Sandulesco, G. (1936). *Helv. chim. acta*, **19**, 1095.
Hobbiger, F. (1955). *Brit. J. Pharmacol.* **10**, 356.
Hobbiger, F. (1956). *Brit. J. Pharmacol.* **11**, 295.
Hobbiger, F. (1957). *Biochim. biophys. Acta*, **25**, 652.
Hobbiger, F., O'Sullivan, D. G. & Sadler, P. W. (1958). *Nature, Lond.*, **182**, 1498.
Hobbiger, F. & Sadler, P. W. (1958). *Nature, Lond.*, **182**, 1672.
Hobbiger, F. & Sadler, P. W. (1959). *Brit. J. Pharmacol.* **14**, 192.
Lands, A. M. & Cavalitto, C. J. (1954). *J. Pharmacol.* **110**, 369.
Michel, H. O. (1958). *Fed. Proc.* **17**, 275.
Somers, L. & Bay, E. (1959). *Fed. Proc.* **18**, 446.
van Eys, J. (1958). *J. biol. Chem.* **233**, 1203.
Wilson, I. B. (1955). *Disc. Faraday Soc.* **20**, 119.
Wilson, I. B., Ginsburg, S. & Quan, C. (1958). *Arch. Biochem. Biophys.* **77**, 286.

Biochem. J. (1960) **75**, 372

The Enzymic Condensation of Porphobilinogen to Porphyrins

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The ability to catalyse the condensation of porphobilinogen to uroporphyrin has been reported for several tissues and extracts. It now appears that the primary product of the enzyme action is uroporphyrinogen, which differs from the porphyrin in having six additional hydrogen atoms. When the reaction is carried out in the presence of oxygen, uroporphyrin is formed by the oxidation of the porphyrinogen.

Although some of the preparations are capable of producing uroporphyrin III from porphobilinogen, all can be so modified that they produce uroporphyrin I only. Bogorad & Granick (1953) obtained *Chlorella* extracts capable of forming uroporphyrin III, together with coproporphyrin III; after heating, the extracts produced uroporphyrin I only. Hoare & Heath (1958) and Heath & Hoare (1959) showed that suspensions of frozen and thawed *Rhodopseudomonas spheroides* as well as the acetone-dried powders from them condense porphobilinogen to uroporphyrin III and coproporphyrin III, but the cell-free supernatants, after centrifuging, and also the heated acetone-dried powders gave uroporphyrin I only. Booij & Rimington (1957) heated vertebrate haemolysates (65° for fowl and 55° for human) which then catalysed the formation of series I porphyrins only. Granick & Mauzerall (1958) found the same for partially purified preparations from rabbit and from fowl erythrocytes. Bogorad (1958*a*, *b*) prepared a preparation from spinach acetone-dried powders which converted porphobilinogen into uroporphyrinogen I. The addition to this of a fraction obtained from wheat-germ extracts, which alone had no action on porphobilinogen, produced a system which formed uroporphyrin III from porphobilinogen.

In this paper, a partially purified preparation from avian erythrocytes, briefly described by Lockwood & Rimington (1957), has been used in a study designed to test proposed mechanisms of the enzymic condensation of porphobilinogen to uroporphyrinogen.

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MATERIALS AND METHODS

Phosphate buffers were made from Na_2HPO_4 and KH_2PO_4. Hydrochloric acid was used for making 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer.

Crystalline porphobilinogen hydrate prepared from the urine of patients with acute porphyria was kindly supplied by Professor C. Rimington. [^{14}C]Formaldehyde was supplied by The Radiochemical Centre, Amersham, Bucks. Fowl blood was collected as described by Dresel & Falk (1954). Calcium phosphate gel was prepared according to Keilin & Hartree (1951). Uroporphyrin I and uroporphyrin III standards were those described by Falk, Dresel, Benson & Knight (1956).

Porphobilinogen estimation. Porphobilinogen was estimated by the method of Rimington, Krol & Tooth (1956). It is not clear from their paper whether the extinction coefficients given refer to the free base, the stable hydrate or to the hydrochloride. Their extinction coefficient ($E^{1\,cm.}_{555\,m\mu}$ 0·114 for a mixture of equal parts of their reagent and a solution containing 1 μg. of porphobilinogen/ml.) agrees with our findings with solutions of known concentration when the porphobilinogen concentration was expressed as the hydrate. This corresponds to molar extinction of 55 500 for the complex.

Porphyrin estimation. Uroporphyrin was estimated in 5% (w/v) HCl solution by measuring the light absorption at 430, 406 and 380 mμ. A molar value of $9{\cdot}50 \times 10^5$ was used for the expression $2E^{1\,cm.}_{406} - E^{1\,cm.}_{430} - E^{1\,cm.}_{380}$ corresponding to a specific value for the expression, 1·143, for a solution containing 1 μg./ml.

Estimation of enzyme activity. To estimate enzyme activity a suitable amount of the solution to be tested was incubated for 1–3 hr. at 37° with 25 μg. of porphobilinogen. The total volume was 0·5 ml. and the solution was 0·05 M with respect to phosphate and at pH 7·3–7·5. The reaction was stopped by adding 0·7 ml. of water and 0·4 ml. of 20% trichloroacetic acid. The proteins were centrifuged off and 1 ml. of the supernatant was diluted to a final volume of 6·4 ml., having 5% (w/v) HCl concentration. Porphobilinogen was estimated in a 2 ml. sample. Under these conditions the colour produced with the aldehyde was stable for at least 5 min. after mixing. The amount of enzyme preparation and the time were chosen so that about half the porphobilinogen disappeared during the incubation. Combined uroporphyrin and uroporphyrinogen were estimated by spectrophotometric measurement of the remainder of the 6·4 ml. dilution after it had been kept in the dark for 25 hr. to allow maximum oxidation of uroporphyrinogen to uroporphyrin. Bogorad (1958*a*) has since shown that higher yields of uroporphyrin are obtained when the oxidation is carried out with iodine than when it is allowed to occur by keeping in air. A small amount of porphyrin also remains adsorbed to the protein. No attempt has been made to correct for either of these losses. Enzyme activities were expressed either as μmole of porphobilinogen consumed/hr. or as μmole of uroporphyrin produced/hr. The latter has been used where possible since porphobilinogen-consumption rates were much more variable with substrate concentration than porphyrin-formation rates.

Isomer type of the uroporphyrins was determined by paper chromatography of the octamethyl ester by the method of Falk & Benson (1953). The uroporphyrin III spot was normally accompanied by the spot ascribed by Falk *et al.* (1956) to *pseudo*uroporphyrin, particularly in the porphyrins from fresh enzyme preparations.

Preparation of enzymes. Fowl erythrocytes were washed twice with 0·9% NaCl and haemolysed with 3 vol. of water. To each 10 vol. of haemolysate, 1 vol. of $CHCl_3$ was added and the mixture was shaken gently on a mechanical shaker for 3–5 hr. The $CHCl_3$ and precipitated haemoglobin were removed by centrifuging, and the solution, which still contained some haemoglobin, was dialysed overnight against running tap water. Small amounts of protein which precipitated during dialysis were removed by centrifuging. The solution was run through a column consisting of cellulose powder and calcium phosphate gel (10 g. of cellulose powder plus 15–20 ml. of gravity-packed gel for each 250 ml. of diffusate; cf. Walker, 1957). Some haemoglobin was retained on the column. The column was then washed with water (about 500 ml.) followed by an equal volume of 0·5% NaCl. This removed much of the haemoglobin and apparently considerable quantities of nucleic acids, together with a small fraction of the enzyme activity. The column was then eluted with phosphate buffer (0·01 M with respect to phosphate in 0·5% NaCl; Na_2HPO_4–KH_2PO_4, 4:1). The enzyme activity was eluted with a narrow coloured band running at the phosphate buffer front. It contained appreciable quantities of haemoglobin or a pigment spectroscopically resembling haemoglobin. From 250 ml. of fowl blood the yield was about 25 ml. of enzyme preparation with an activity of 0·2–0·4 μmole of porphyrin/ml./hr. with a protein content of about 20 mg./ml. For some purposes this solution was concentrated by pressure-filtration through Viscose tubing as demonstrated by Grant, Rowe & Stanworth (1958). This gave a thick syrupy liquid without significant loss of activity.

Human erythrocytes contain the same enzyme system but it is less stable; particularly it easily loses its ability to produce uroporphyrin III; this is exemplified by sensitivity to heat. After heating at 50° for 15 min. the enzyme system from human erythrocytes is capable of producing uroporphyrin I only, whereas that from fowl erythrocytes appears not to be affected by this treatment. Acetone precipitation either of the erythrocytes or of the haemolysate gave an active dried powder (although occasionally a preparation in which uroporphyrin I and not III was produced), but although there was little, if any, loss of activity in preparing the acetone-dried powder, the precipitation achieved no purification. Little purification was achieved by $(NH_4)_2SO_4$ precipitation because the enzyme activity precipitated in the same range as the haemoglobin, e.g. during $(NH_4)_2SO_4$ precipitation of an acetone-dried powder extract of human erythrocytes 15% of the total activity came down at 40% saturation, 35% at 50% saturation and 5% at 55% saturation; the remaining activity was lost. Some separation could be achieved but the enzyme became increasingly sensitive to dialysis as the purification proceeded. Ethanol–$CHCl_3$ precipitation of the haemoglobin was very destructive of enzyme activity.

The preparation described produces from porphobilinogen uroporphyrin only. It appears equally active in the presence or absence of oxygen. In the absence of oxygen no uroporphyrin is formed but a precursor which gives uroporphyrin on oxidation in air. This precursor is believed to be uroporphyrinogen; on exposure to air there is first an increase of absorption at 500 mμ followed by a decrease as

the porphyrin spectrum develops (Bogorad, 1958*a*, *b*, and Granick & Mauzerall, 1958). All experiments described have been carried out in air. The ability to form coproporphyrin (or coproporphyrinogen) is completely lost after treatment of the haemolysate with $CHCl_3$. Short treatment of chicken erythrocytes with benzene produces a haemolysate which can no longer synthesize protoporphyrin, but which still retains the ability to synthesize coproporphyrin (coproporphyrinogen). The preparation was stable for several weeks. There seemed little difference in the stability at room temperature, at 2° and at −10°.

RESULTS

Effects of heat. The results of heat-treatment of the enzyme preparations were quantitatively similar to the findings of Booij & Rimington (1957). The human enzyme was the more sensitive; after heating at 55° for 15 min. and at pH 7·4, it yielded with porphobilinogen uroporphyrin I only. The fowl enzyme was more resistant; after heating at 55° for 15 min. at pH 7·4, it still produced uroporphyrin III but no detectable amounts of uroporphyrin I. When heated at 60–62° the enzyme appeared to be completely altered after 10 min., giving no detectable amounts of uroporphyrin III. After heating for 2 min. or 5 min. there was partial alteration of the enzyme system. The sensitivity of the system to heating was influenced by pH; heating a fowl enzyme system for 15 min. at 55° at pH 8·5 completely altered it to a uroporphyrin I-producing system. To produce an altered (uroporphyrin I-producing) system for further studies the intact system was heated at 60–62° for 15 min. at pH 7·4.

Heating the enzyme preparation at 65° or less had little effect on the over-all rates of porphyrin production and porphobilinogen consumption. At higher temperatures there was significant loss of enzyme activity. Table 1 gives the results of heating at 55° and at 60°. The slight increase in activity was often, but not invariably, found. It agrees with the findings of Booij & Rimington (1957), who observed an increase in the rate of total porphyrin formation in fowl erythrocytes after heating at 65°.

Table 1. *Effect of heat on activity of enzyme preparation*

Enzymic activity was estimated in 0·11 M-tris buffer, pH 8·3, with porphobilinogen 0·13 mM at 37°. The limits given for the accuracy of the values express the errors of the spectrophotometric readings and do not include losses and other systematic errors in the method of estimation.

Treatment		Subsequent activity (% of activity of unheated enzyme preparation)	
Time (min.)	Temp.	Porphobilinogen consumption	Porphyrin formation
5	55°	100±3	100±1
10	55	100±3	106±1
30	55	102±3	99±1
5	60	106±3	115±1
10	60	102±3	114±1
30	60	92±3	91±1

Incorporation of formaldehyde. Shemin, Abramsky & Russell (1954) isolated [^{14}C]formaldehyde, formed from porphobilinogen, labelled with ^{14}C in the amino methyl group, during enzymic condensation of the porphobilinogen to porphyrin. We were interested to investigate whether [^{14}C]formaldehyde would be incorporated into porphyrin during the enzymic condensation of non-radioactive porphobilinogen. [^{14}C]Formaldehyde (1·4 μg. with an activity of 0·05 μc) was incubated at 37° for 10 hr. with porphobilinogen hydrate (25 μg.) and excess of enzyme in tris buffer, pH 8·5 (total volume 0·55 ml.). A tube containing the same quantities of porphobilinogen and formaldehyde in 0·1 N-HCl but without enzyme was heated for 15 min. in a boiling-water bath. The times were sufficient to give maximum yield of porphyrin in both tubes. As a control, porphobilinogen and formaldehyde were incubated at 37° in tris buffer, but without enzyme. At the end of the 10 hr. a very faint fluorescence showed negligible porphyrin production in the control tube. Formaldehyde inhibits the enzyme but at the concentration used the inhibition cannot be detected. The uroporphyrin from the two tubes (enzymically-formed porphyrin and chemically-formed porphyrin) was isolated, esterified and crystallized. Carrier formaldehyde (1 mg.) was added to the washings obtained during the isolation of the enzymically-formed porphyrin and the dimedone compound was isolated and counted at infinite thickness. A count of 32 000 counts/min. showed that formaldehyde was still available in the enzyme solution at the end of the experiment. The porphyrin was prepared for counting by allowing approx. 4 μg. in chloroform solution to dry on a lens-tissue circle on the planchet; this approximates to an infinitely thin layer. The background count was 13 counts/min., giving a correction for background, for the counts done on 4 μg. samples, of −3 counts/min./μg. The activity of the enzymically-prepared porphyrin was not significantly different from the background (1 count/min./μg. after correction). The chemically-prepared porphyrin gave a corrected count of 385 counts/min./μg. In another experiment carried out at pH 7·4 the count of the enzymically-prepared porphyrin (31 counts/min./μg.) was not negligible, although less than a tenth of that of the chemically-prepared porphyrin (405 counts/min./μg.). Spontaneous porphyrin formation increases with decrease of pH. In a radioautograph of a chromatogram of the chemically-prepared porphyrin most of the activity coincided exactly with

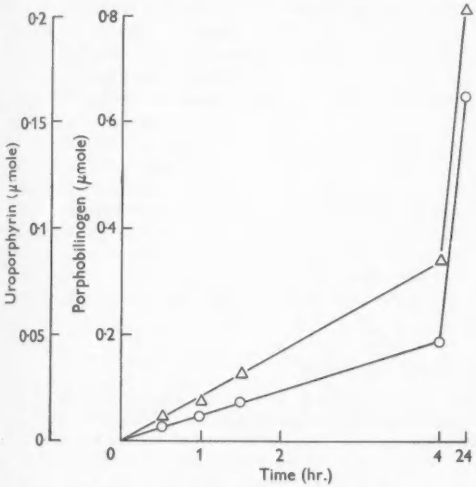


Fig. 1 (*a*). Porphobilinogen consumption and uroporphyrin formation by intact enzyme. △, Porphobilinogen consumed; ○, porphyrin formed. System contained: 0·4 ml. of intact enzyme, 0·88 μmole of porphobilinogen, phosphate buffer, pH 7·4 (final concn. 0·05 M), to 4 ml. Temperature 37°. Samples (0·5 ml.) were taken for analysis.

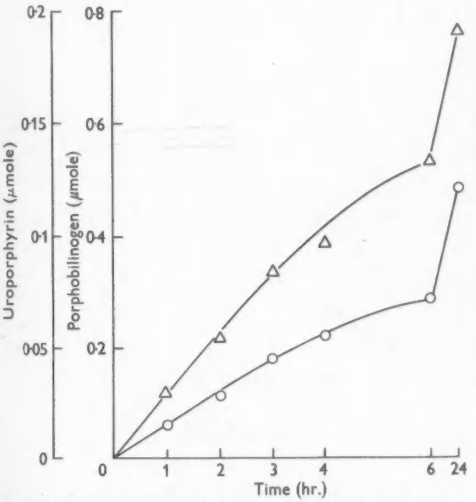


Fig. 1 (*b*). Porphobilinogen consumption and uroporphyrin formation by heated enzyme. △, Porphobilinogen consumed; ○, porphyrin formed. System contained: 0·2 ml. of enzyme which had been heated for 30 min. at 60°, 0·78 μmole of porphobilinogen, tris buffer, pH 8·3 (final concn. 0·04 M), to 5 ml. Temperature 37°. Samples (0·5 ml.) were taken for analysis.

the uroporphyrin III methyl ester spot, although some activity remained at the point of application. Enzymically-prepared porphyrins, both isomer III from intact enzyme and isomer I from heat-altered enzyme, showed on the radioautographs only faint traces of activity. The traces coincided with the respective porphyrin spots.

Rate of porphyrin formation. Figs. 1 (*a*) and 1 (*b*) show porphobilinogen consumption and porphyrin production for both intact and heat-altered enzyme preparations. Both heated and unheated enzyme preparations show initially linear porphyrin production. There is no evidence of any lag. Porphobilinogen consumption is more irregular.

Dependence of pH. Fig. 2 (*a*) shows porphyrin formation by both the intact and the heat-altered enzyme systems as a function of pH. Fig. 2 (*b*) shows the same for porphobilinogen consumption. At lower pH values there is appreciable porphobilinogen consumption without porphyrin production but elsewhere the shape of the four curves is identical within the limits of experimental error. The maximum of pH 8·0–8·5 reported by Lockwood & Rimington (1957) applies to the human enzyme system. The maxima found and the shape of the curve for the fowl enzyme system agree with those reported by Granick & Mauzerall (1958).

Michaelis constants. Michaelis constants were calculated on porphobilinogen consumption. For the intact preparation values of 8, 9, 15, 17, 19 and 19 μM were obtained and for the heat-altered preparation values of 6, 6, 8, 11 and 19 μM. The value decreased on storage of the enzyme preparation. When both estimations were carried out on the same preparation, heat treatment caused a decrease in the Michaelis constant; thus pairs of values before and after heat treatment were 19 and 8 μM respectively and, for another preparation, 17 and 11 μM. If this increase in affinity for the enzyme is not accompanied by a change in the reaction rate of the enzyme–substrate complex (namely, if $V_{max.}$ remains constant) it could explain the slight increase in the activity of the heat-treated enzyme shown in Table 1. The activities quoted in Table 1 were measured at a substrate concentration of 0·2 mM. A decrease of K_m from one-tenth of the substrate concentration to one-twentieth should change the rate from 0·9 $V_{max.}$ to 0·95 $V_{max.}$. Apparent values of the Michaelis constant based on porphyrin formation were higher than those based on porphobilinogen consumption. Figs. 3*a* and 3*b* show graphs used to calculate the Michaelis constants. Porphyrin-production values are also plotted for comparison.

Porphyrin yield. Yields of porphyrin based on porphobilinogen consumption varied from 50 to 90 % of the theoretical required for the formation

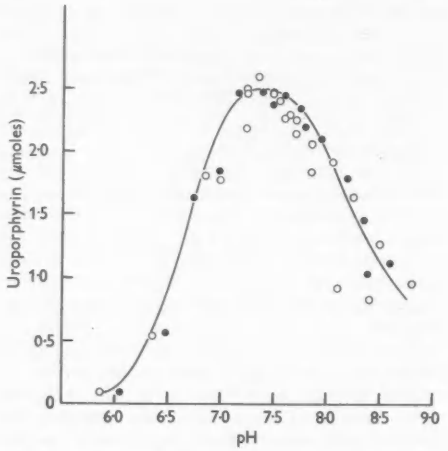


Fig. 2 (*a*). Effect of pH on porphyrin formation. System contained: enzyme, 0·2 ml. and porphobilinogen, 0·4 μmole. Incubation was at 37° for 2 hr. Volume, 10 ml. Phosphate and tris buffers of final concentrations 0·04 and 0·03 M respectively were used. ○, Intact enzyme; ●, heated enzyme; the intact and heated enzymes did not come from the same batch. The estimate is in terms of maximum activity, excluding some apparently aberrant estimations. Half the volume was used for pH measurement. The other half was incubated. A portion (5 ml.) was used for analysis as given in the Materials and Methods section except that 2 ml. of trichloroacetic acid and 1 ml. of 11 N-HCl were added without further dilution.

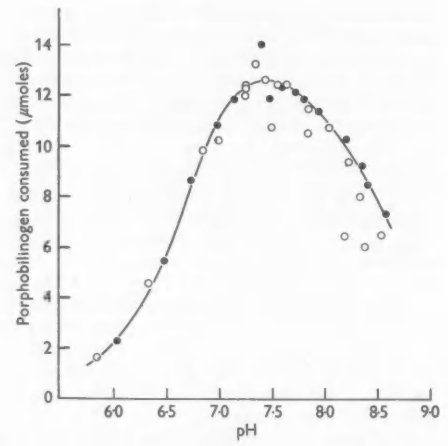
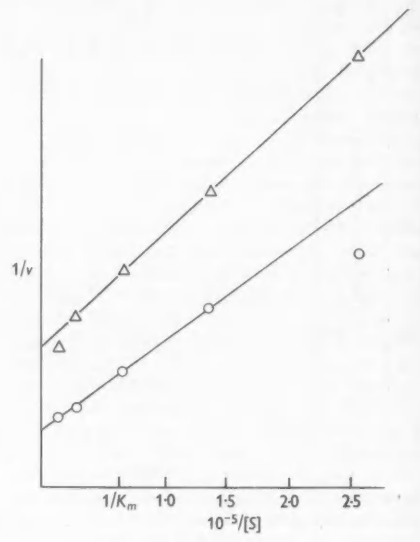
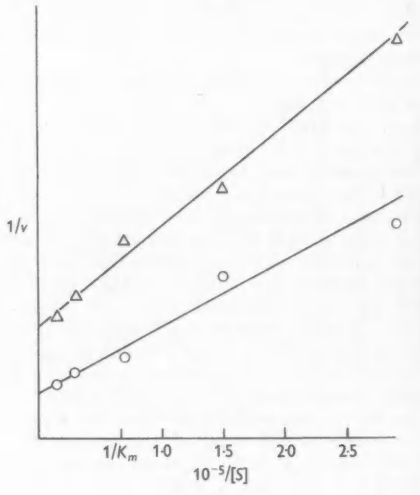


Fig. 2 (*b*). Effect of pH on porphobilinogen consumption. Details were as given in Fig. 2 (*a*).





Figs. 3 (*a*) and 3 (*b*). Curves used for determination of Michaelis constant. (*a*) Intact enzyme. (*b*) Enzyme which had been heated at 60° for 30 min. Tris buffer, pH 8·3 (final concn. 0·03 M). Volume of each system, 5 ml.; enzyme 0·05–0·2 ml. with increasing substrate concentration; incubation was at 37° for 2 hr. ○, Porphobilinogen-consumption rates; △, porphyrin-formation rates. Ordinate in arbitrary units.

of 1 molecule of porphyrin from 4 molecules of porphobilinogen. Yields above 80%, that is less than 5 molecules of porphobilinogen/molecule of porphyrin for both intact and heat-altered enzyme were obtained in experiments in which the porphyrin formation was rapid.

Inhibitors. In Table 2 are shown the effects of possible inhibitors. The inhibition by metal ions such as copper, cobalt, etc., occurs at relatively high concentrations and probably has little significance. Table 3 shows that Hg^{2+} ions inhibit and the inhibition is reversed by sulphide. The system is also inhibited by *p*-chloromercuribenzoate and the inhibition is reversed by 1:2-dimercaptopropan-3-ol. The reversible inhibition by mercury compounds is evidence that the system depends on SH groups for its activity. Systems partially inhibited by Co^{2+} ions and systems in which *p*-chloromercuribenzoate inhibition has been reversed by dimercaptopropanol produce isomer III. It can be seen from the Table that there is no inhibition by CN^- or N_3^- ions. However, in the presence of either cyanide or azide (mM) the enzyme system produces isomer I only. The system was slightly inhibited by relatively high concentrations of formaldehyde and of dimedone.

Paper electrophoresis. Attempts were made further to purify the enzyme system by paper

Table 2. *Action of some common inhibitors*

Each tube contained inhibitor, 0·1 ml. of intact enzyme preparation, tris buffer, pH 8·3 (0·03 M final concentration), 0·1 μmole of porphobilinogen. The porphobilinogen was added 5 min. after the addition of the inhibitor. Total volume, 0·5 ml. Incubation was at 37° for 3 hr. Estimations of porphobilinogen and porphyrins were as described in the Materials and Methods section.

Inhibitor	Concn. (mM)	Rate (% of control without inhibitor) Porphobilinogen consumption	Porphyrin formation
Co^{2+}	0·03	100*	83*
	0·2	87	57
Zn^{2+}	0·03	100*	104*
	0·2	35	15
Ni^{2+}	0·03	100*	102*
	0·2	82	87
Cu^{2+}	0·03	100*	91*
Pb^{2+}	0·03	100*	102*
Hg^{2+}	0·002	109	99
	0·02	5	0
	0·02	—	4
	0·2	—	3
	0·2	0	1
	0·1	0	0
Chloromercuribenzoate	0·1	22	1
	1	7	1
S^{2-}	0·01	96	95
	0·1	99	96
	0·1	—	97
	1	89	78
	1	—	77
	1	100*	81*
Dimercaptopropanol	0·1	98	100
	1	92	108
	2	102	118
Iodoacetate	1	98	84
CN^-	1	109	109
	1	100*	94*
N_3^-	1	98	91
	1	100*	94*
F^-	1	100	89
	1	100*	96*
AsO_2^-	1	104	97
	1	100*	92*

* The substrate in these experiments was completely exhausted in the control without inhibitor.

Table 3. *Reversal of inhibition*

Conditions were as given for Table 2. The sulphide and the dimercaptopropanol were added 5 min. after the addition of the inhibitor.

Substance	Concn. (mM)	Rate (% of control without test substance)	
		Porphobilinogen consumption	Porphyrin formation
S^{2-}	1	—	77
Hg^{2+}	0·2	—	3
$Hg^{2+} + S^{2-}$	As above	—	8
S^{2-}	0·1	—	97
Hg^{2+}	0·02	—	4
$Hg^{2+} + S^{2-}$	As above	—	89
Dimercaptopropanol	2	102	118
Chloromercuribenzoate	1	7	1
Dimercaptopropanol + chloromercuribenzoate	As above	89	90
Chloromercuribenzoate	0·1	22	1
Dimercaptopropanol + chloromercuribenzoate	As above	96	101

electrophoresis. It was thought that if there were an independent (but in the intact system, inhibited) enzyme which forms uroporphyrin I, it might be possible after electrophoresis to obtain an active enzyme from the intact system which no longer gave a uroporphyrin I-forming enzyme after heating. The possibility of obtaining a separation of a condensing (uroporphyrin I-forming) enzyme and an isomerizing enzyme similar to those described by Bogorad (1958*a*, *b*) was also considered. The enzyme was run on Whatman 3 MM paper on a water-cooled apparatus at potential pressures varying from 15 to 50 v/cm. Buffers used were phosphate, pH 6·8 and 7·4, concentration 0·01–0·1 M. Protein staining showed the development of a continuous pattern with haemoglobin-like compounds accumulating at the anodic end of the streak (these components hardly moved at pH 6·8 and moved slightly anodically at pH 7·4). To test for enzyme activity the paper was sprayed with porphobilinogen solution (500 μg./ml.) in 0·2 M-phosphate buffer, pH 7·4, and incubated for 3–15 hr. at 37° in a glass jar containing a beaker with a little water to keep the paper moist. Several fluorescent spots developed. Those developing on the electrophoresis papers of heat-altered preparations had moved further cathodically than those of the intact preparations. Porphyrin isolated from the fluorescent spots that developed on the electrophoresis papers of the heat-altered preparations proved, on paper chromatography, to be uroporphyrin I; from the intact system a mixture of uroporphyrins I and III was isolated. Although pure isomer III was not produced no apparent difference could be found in the ratio of the isomers in the porphyrin isolated from different spots. At pH 7·4 an anodic spot of enzyme activity appeared regularly in the electrophoresis paper in a position where there was no detectable activity in the electrophoresis paper of the heat-altered enzyme. Two parallel electrophoresis papers of intact preparation were prepared: in one the spot was identified by spraying with porphobilinogen and from the other the spot was cut out, the circle of paper was suspended in 0·05 M-phosphate buffer, pH 7·5, heated for 15 min. at 60° and then incubated with porphobilinogen. The porphyrin was isolated and shown to be uroporphyrin I by paper chromatography.

DISCUSSION

In the enzyme system described by Bogorad (1958*a*, *b*) the enzyme responsible for the formation of uroporphyrin I is part of the system which forms uroporphyrin III, and Bogorad finds that uroporphyrin I-forming enzyme is an essential part of his reconstituted uroporphyrin III-forming system. This appears to be true also for the system from *Rhodopseudomonas spheroides* as described by Heath & Hoare (1959).

In this paper it has been shown that an avian uroporphyrin III-forming system can be converted into one forming uroporphyrin I with the same pH curve and having very similar affinity for the substrate, and particularly that the overall rate of porphyrin formation remains unchanged after alteration either by heating or by cyanide or azide. We are therefore confident in claiming that in these systems there is no independent uroporphyrin I-forming enzyme, such as an enzyme associated with a heat-labile inhibitor.

From the results with [^{14}C]formaldehyde it is possible to exclude the participation of free formaldehyde in the formation of either uroporphyrin I or uroporphyrin III. The evidence does not exclude the transfer of bound formaldehyde

from porphobilinogen to an enzyme and thence to the free α-position of another molecule of porphobilinogen or to that of an intermediate, but does require that such an enzyme should be incapable of reacting with free formaldehyde.

Bogorad (1958*a*, *b*), using good methods of estimation of combined uroporphyrinogen and uroporphyrin, has shown that less than 4·5 molecules of porphobilinogen are required for the synthesis of 1 molecule of uroporphyrin I or III. Granick & Mauzerall (1958) have shown the same for uroporphyrin III formation by avian enzyme. With methods of porphyrin estimation involving greater losses, we have nevertheless shown that under favourable conditions less than 5 molecules of porphobilinogen are necessary for the formation of either porphyrin isomer.

Bogorad finds that the formation of uroporphyrin I by spinach enzyme and the formation of uroporphyrin III by the reconstituted enzyme are as active in the absence of oxygen as in its presence. The enzyme described in this paper was also found to be active in the absence of oxygen. This fits with findings by several workers, Neve, Labbe & Aldrich (1956), Hoare & Heath (1958), Mauzerall & Granick (1958), that uroporphyrinogen and not uroporphyrin is the substrate for the decarboxylase (uroporphyrinogen decarboxylase), which is the next enzyme in the pathway of protoporphyrin biosynthesis.

It is now possible to make certain postulates for the enzymic condensation of porphobilinogen to porphyrins.

(1) In the formation of both isomers there occurs the same primary enzymic reaction with porphobilinogen.

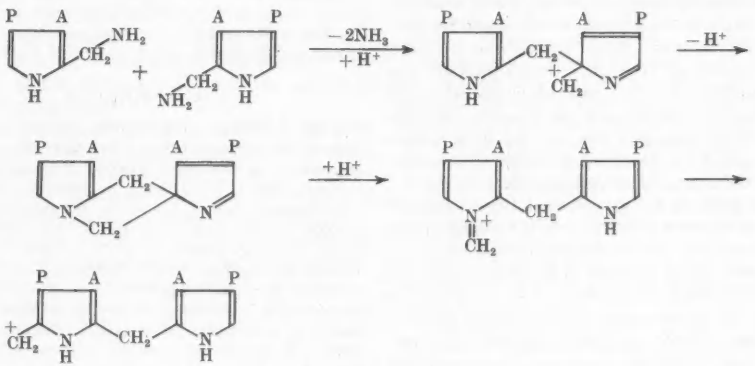
(2) The primary reaction is rate-limiting in both systems.

(3) Free formaldehyde does not participate in the enzymic reaction, although the evidence does not exclude the possibility that an enzyme, incapable of reacting with free formaldehyde, conveys bound formaldehyde from one pyrrole nucleus to another.

(4) Four molecules of porphobilinogen only are required for the formation of either isomer of uroporphyrin.

(5) No oxidation occurs until after cyclization to porphyrinogen and therefore any intermediates formed are in the reduced form, such as dipyrromethanes, not dipyrromethenes.

If these postulates are accepted it is possible immediately to reject certain proposed mechanisms. That proposed by Shemin (1955) requires the loss of one porphobilinogen unit, for example as opsopyrrole dicarboxylic acid [3(4)-carboxymethylpyrrole-4(3)-β-propionic acid]. The findings of Carpenter & Scott (1959), who showed that labelled opsopyrrole dicarboxylic acid is not incorporated into porphyrin during biosynthesis, make unlikely any modification of this scheme whereby opsopyrrole dicarboxylic acid acts as an intermediate or as a cofactor of an isomerizing enzyme. Rimington (1955) has pointed out that the mechanism of the Corwin–Andrews reaction with formation of a tripyrromethane can apply only to the dipyrromethenes, not to the dipyrromethanes, an argument against the mechanisms proposed by Shemin (1955) and by Granick (1955). That proposed by Shlyk (1956) with the participation of a porphobilinogen aldehyde would necessitate either the loss of one pyrrole unit or the formation of a dipyrromethene as an intermediate, and would also



P is $-CH_2 \cdot CH_2 \cdot CO_2H$
A is $-CH_2 \cdot CO_2H$

Scheme 1

require an oxidation step for the formation of the aldehyde.

Bullock, Johnson, Markham & Shaw (1958) have proposed the mechanism shown in Scheme 1 to explain the chemical formation of uroporphyrin III rather than I in acid solution.

They propose two repetitions of the same condensation with the formation successively of a tripyrrolic chain ($^{+}CH_2 \cdot PA \cdot PA \cdot AP$) and a tetrapyrrolic chain ($^{+}CH_2 \cdot PA \cdot PA \cdot PA \cdot AP$), where P is $-CH_2 \cdot CH_2 \cdot CO_2H$ and A is $-CH_2 \cdot CO_2H$, and claim that ring closure would then give uroporphyrinogen III. The mechanism could apply equally well to the enzymic formation of uroporphyrinogen. If there is immediate ring closure on the formation of a tetrapyrrolic chain (A, below) then uroporphyrinogen III would be the only isomer formed. If, however, ring closure does not occur immediately on the formation of the tetrapyrrolic chain and a longer polypyrrolic chain is formed, still attached, however, to the enzyme surface, then ring closure could give 1 molecule of uroporphyrinogen III followed by an indefinite number of molecules of uroporphyrinogen I.

(A) $E + 4\ pbg \rightarrow E \cdot CH_2 \cdot PA \cdot PA \cdot PA \cdot AP \rightarrow E + \text{uroporphyrinogen III}$

(B) $E + 5\ pbg \rightarrow E \cdot CH_2 \cdot PA \cdot PA \cdot PA \cdot PA \cdot AP \rightarrow E \cdot CH_2 \cdot PA + \text{uroporphyrinogen III}$

$E \cdot CH_2 \cdot PA + 4\ pbg \rightarrow E \cdot CH_2 \cdot PA \cdot PA \cdot PA \cdot PA \cdot PA \rightarrow E \cdot CH_2 \cdot PA + \text{uroporphyrinogen I}$

(pbg, Porphobilinogen).

There is no reason why the ring closure suggested in A should not be enzymic and heat- and cyanide-sensitive. Heat would then convert the system from one forming uroporphyrinogen III into one forming uroporphyrinogen I with a small fraction of the III isomer. Such mechanisms would be consistent with the similarity of pH-dependence and Michaelis constants and they could explain the constancy of the reaction rate after alteration of the system by heating and by cyanide. In the terms of this mechanism the enzyme from wheat germ described by Bogorad (1958*b*) as an 'isomerase' would be a cyclizing enzyme.

Bogorad (1958*b*) has shown that the completed system which forms uroporphyrin III consists of at least two enzymes. No evidence has been obtained in the present work of more than one enzyme; in particular electrophoresis showed no evidence of separation into a condensing enzyme with porphobilinogenase activity and an enzyme with 'isomerizing' (or cyclizing) activity. However, the mechanism proposed might be catalysed by two distinct enzymes, such as those described by Bogorad, or it might be catalysed by a single enzyme possessing both forms of activity.

SUMMARY

1. An enzyme preparation from fowl erythrocytes is described which condenses porphobilinogen to uroporphyrinogen III. After heating the preparation at 60° it forms only uroporphyrinogen I. The reactions with porphobilinogen of the intact and of the heat-altered preparation are compared. Formation of porphyrinogen was measured as the porphyrins formed by their oxidation in air.

2. Neither the intact nor the heat-altered preparation incorporates free formaldehyde into porphyrin.

3. Initial porphyrin formation by both preparations is linear with time.

4. Substrate affinity and pH-dependence of the preparation is not appreciably altered by heat treatment.

5. Mercuric and *p*-chloromercuribenzoate ions inhibit the intact preparation completely. The inhibition is reversed by sulphide ions and by dimercaptopropanol respectively. The isomer formed after reversal of the *p*-chloromercuribenzoate inhibition is uroporphyrin III.

6. Cyanide ions do not inhibit the intact preparation but in the presence of cyanide the intact preparation forms uroporphyrin I.

7. Neither heat treatment nor the presence of cyanide appreciably influences the rate of porphyrin formation.

8. Paper electrophoresis failed to show any separation into fractions with different types of enzyme activity.

9. The findings are discussed in terms of possible mechanisms of porphyrin formation from porphobilinogen.

We are indebted to Professor C. Rimington, F.R.S., in whose Laboratory this work was done. One of us (W.H.L.) wishes to thank the Nuffield Foundation for a Nuffield Dominions Travelling Fellowship and also the National Health and Medical Research Council of Australia.

REFERENCES

Bogorad, L. (1958*a*). *J. biol. Chem.* **233**, 501.
Bogorad, L. (1958*b*). *J. biol. Chem.* **233**, 510.
Bogorad, L. & Granick, S. (1953). *Proc. nat. Acad. Sci., Wash.*, **39**, 1176. Quoted in Granick (1955). *Ciba Foundation Symp., Porphyrin Biosynthesis and Metabolism*, p. 146. London: J. and A. Churchill Ltd.
Booij, J. L. & Rimington, C. (1957). *Biochem. J.* **65**, 4P.
Bullock, E., Johnson, A. W., Markham, E. & Shaw, K. B. (1958). *J. chem. Soc.* p. 1430.
Carpenter, A. T. & Scott, J. J. (1959). *Biochem. J.* **71**, 326.
Dresel, E. I. B. & Falk, J. E. (1954). *Biochem. J.* **56**, 156.
Falk, J. E. & Benson, A. (1953). *Biochem. J.* **55**, 101.
Falk, J. E., Dresel, E. I. B., Benson, A. & Knight, B. C. (1956). *Biochem. J.* **63**, 87.
Granick, S. (1955). *Ciba Foundation Symp., Porphyrin Biosynthesis and Metabolism*, p. 147. London: J. and A. Churchill Ltd.

Granick, S. & Mauzerall, E. (1958). *J. biol. Chem.* **232**, 1119.
Grant, G. H., Rowe, D. S. & Stanworth, D. R. (1958). *Biochem. J.* **69**, 13P.
Heath, H. & Hoare, D. S. (1959). *Biochem. J.* **72**, 13.
Hoare, D. S. & Heath, H. (1958). *Nature, Lond.*, **181**, 1592.
Keilin, D. & Hartree, E. F. (1951). *Biochem. J.* **49**, 88.
Lockwood, W. H. & Rimington, C. (1957). *Biochem. J.* **67**, 8P.
Mauzerall, D. & Granick, S. (1958). *J. biol. Chem.* **232**, 1141.
Neve, R. A., Labbe, R. F. & Aldrich, R. A. (1956). *J. Amer. chem. Soc.* **78**, 691.
Rimington, C. (1955). *Rep. Progr. Chem.* **51**, 311.
Rimington, C., Krol, S. & Tooth, B. (1956). *Scand. J. clin. Lab. Invest.* **8**, 251.
Shemin, D. (1955). *Ciba Foundation Symp., Porphyrin Biosynthesis and Metabolism*, p. 18. London: J. and A. Churchill Ltd.
Shemin, D., Abramsky, T. & Russell, C. S. (1954). *J. Amer. chem. Soc.* **76**, 1204.
Shlyk, A. A. (1956). *Metod mechenykh Atomov v izuchenii biosinteza khlorofilla*, p. 248. Minsk: Izdatelstvo Akademii Nauk, B.S.S.R.
Walker, D. J. (1957). *Biochem. J.* **67**, 4P.

Biochem. J. (1960) **75**, 381

Studies on Flavinadenine Dinucleotide-Synthesizing Enzyme in Plants

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There is practically no information on the biosynthesis of flavinadenine dinucleotide in plants. The available evidence relating to this process in animal tissues or micro-organisms is meagre, although the function of flavin nucleotides as components of respiratory enzymes has long been known. Trufanov (1941) suggested that flavinadenine dinucleotide is synthesized in rat-tissue slices by a condensation of riboflavin and adenosine diphosphate and that maintenance of cell structure is essential for the reaction. Schrecker & Kornberg (1950) demonstrated synthesis of flavinadenine dinucleotide from flavin mononucleotide and adenosine triphosphate in yeast. A similar pathway for synthesis of flavinadenine dinucleotide was reported in a riboflavin-secreting mutant yeast (Giri & Krishnaswamy, 1956). Recently Deluca & Kaplan (1958) showed that synthesis of flavinadenine dinucleotide occurs in rat-liver supernatants.

The occurrence of riboflavin in plants has long been known (Bonner, 1942; Bonner & Bonner, 1948; Watson & Nogelle, 1947), but there is no information on the concentration of flavin nucleotide coenzymes and the enzymes synthesizing these.

After the observation on the widespread occurrence of the flavin nucleotides (Giri, Appaji Rao, Cama & Kumar, 1959) and on the biosynthesis of flavin mononucleotide in plants by phosphorylation of riboflavin by adenosine triphosphate (Giri, Krishnaswamy & Appaji Rao, 1958), the mechanism of biosynthesis of flavinadenine dinucleotide was studied, and the results are described and discussed in this paper.

* Deceased.

MATERIALS AND METHODS

Substrates and reagents. Flavin mononucleotide (FMN) was a Hoffman–La Roche and Co. product obtained as a gift from Voltas Ltd., Bombay, and was purified before use. Flavinadenine dinucleotide (FAD; 60% pure) was obtained as a gift from Esai Ltd., Japan, and was purified by preparative circular-paper chromatography before use (Giri, 1954, 1955). Adenosine triphosphate (ATP) used included preparations made according to LePage (1949) of 65–70% purity and another (95% purity) obtained from California Foundation for Biochemical Research. The purity of ATP was determined by the method described by LePage (1949). All other chemicals used were of reagent grade.

Preparation of alumina Cγ-gel. Ammonium sulphate (22 g.) was dissolved in 600 ml. of water. Ammonia soln. (100 ml. of 10%; previously standardized by titration) was added to it and the mixture was warmed to 58°. A solution containing 76·7 g. of aluminium ammonium sulphate dodecahydrate in 150 ml. of water was also warmed to 58° and was added all at once to the basic ammonium sulphate solution. This mixture was warmed to 60° and stirred for 15 min. The contents were transferred to a tall jar with 5 l. of water and the precipitate was washed three times with 3 l. of water. To the fourth wash was added 80 ml. of 20% ammonia soln. to decompose the basic sulphate and the gel was washed repeatedly with water. After twenty washings the water remained opalescent and the gel was washed twice more with water. A suspension containing 18 mg. (dry wt.)/ml. was prepared. It was aged at 4° for at least 2 months before use (Willstätter & Kraut, 1923).

Preparation and purification of the enzyme. Finely powdered green gram (*Phaseolus radiatus*) was used as the source of the enzyme. An extract with maximum specific activity was obtained by the following procedure.

Freshly powdered green-gram seeds (100 g./300 ml.) were extracted with 0·1 M-sodium bicarbonate soln. with constant stirring at 0–5° for 2 hr. The extract was centrifuged

at 1000 *g* for 15 min. and the supernatant (stage I, Table 1) was dialysed against glass-distilled water for 18 hr. at 0–5°. The diffusate was centrifuged at 2000 *g* for 15 min. (stage II), the supernatant was brought to 0·45 saturation with ammonium sulphate by the addition of recrystallized solid ammonium sulphate and the precipitate obtained on centrifuging at 5000 *g* was rejected. The supernatant was raised to 0·55 saturation with ammonium sulphate, the precipitate obtained on centrifuging at 5000 *g* for 30 min. was dissolved in glass-distilled water and was dialysed free from ammonium sulphate (stage III). To the dialysed extract (40 ml.) was added 4 ml. of ethanol at 0° to −5° and the precipitate obtained on centrifuging was rejected. To the supernatant 5·5 ml. of ethanol was added and the precipitate obtained on centrifuging at 5000 *g* for 20 min. was dissolved in 35 ml. of glass-distilled water (stage IV). An equal volume of alumina C*γ*-gel (18 mg./ml.) was added and the enzyme–gel mixture was kept cold for 20 min. with constant stirring, centrifuged at 1000 *g* for 5 min. and the supernatant was rejected. The gel was extracted three times with 10 ml. volumes of 0·02 M-sodium phosphate buffer (pH 7·8) and the eluates were combined. The combined eluates were precipitated with ethanol, to 8% concentration, centrifuged at 5000 *g* for 45 min. and the supernatant was rejected. The precipitate was dissolved in 20 ml. of glass-distilled water and used as the enzyme (stage V). An 83-fold purification with 26% recovery was achieved.

Enzyme assay. During assay and analysis, tubes (1 cm. × 10 cm.) in which the reactions were carried out were protected from light. The reaction mixture contained: FMN, 1 mM; ATP, 20 mM; NaF, 0·1 M; $MgSO_4$, 0·1 mM; veronal–HCl buffer (pH 7·4), 0·1 M; 0·8 ml. of the enzyme preparation; total volume, 2 ml. Reaction was stopped by heating in boiling water for 20 min. The tubes were centrifuged at 3000 *g* for 20 min. and suitable portions (usually 0·3 ml.) were analysed for FAD by the circular-paper-chromatographic technique (Giri & Krishnaswamy, 1956), butanol–acetic acid–water (4:1:5, by vol.) being used as the solvent system. The FAD band was located under u.v. light and marked with a pencil. The band was eluted into 5 ml. of glass-distilled water for 6 hr. and fluorescence of the eluates measured in a Klett fluorimeter with filters (*a*) at the lamp source, Corning 5113 + 3389 (4358 Å), and (*b*) at the photocell, Corning 3486 (5200 Å). The necessary blanks and controls were always run.

Protein was determined by the biuret method of Robinson & Hogden (1940). One unit of enzyme activity is defined as the amount that synthesizes 1 μm-mole of FAD at pH 7·4, temperature 37°, in 60 min.

For preparative purposes the technique of Giri (1954, 1955) was used. Absorption spectra were determined in a Beckman spectrophotometer model DU.

Determination of flavinadenine dinucleotide in seedlings. FAD in germinating seedlings was determined by the method of Bessey, Lowry & Love (1949). The seeds were homogenized and the homogenates deproteinized with trichloroacetic acid. The deproteinized extracts were neutralized and fluorescence was measured in a Klett fluorimeter with appropriate filters. Another portion of the deproteinized extract was hydrolysed by incubating at 37° for 18 hr., and it was neutralized and the fluorescence was measured in a Klett fluorimeter. With the equation suggested by Bessey *et al.* (1949), the FAD content of the portions was determined.

RESULTS

Identification of enzymically synthesized flavinadenine dinucleotide. A 20 ml. reaction mixture was incubated at 37° for 18 hr. and the reaction stopped as indicated above. The concentrated extract was chromatographed and the flavin band (R_F 0·18) was eluted into glass-distilled water. The eluate showed absorption maxima at 263, 375 and 450 mμ and the spectrum was identical in all respects with that of an authentic sample of FAD. The eluate, on chromatography in four different solvent systems [(i) butan-1-ol–acetic acid–water (4:1:5, by vol.); (ii) butan-1-ol–propanol–water (2:2:1, by vol.); (iii) butan-1-ol–formic acid–water (77:10:13, by vol.); (iv) *iso*amyl alcohol saturated with water], was identical with an authentic sample of FAD. Cochromatography in all these solvent systems was carried out and the authentic sample moved as a single band with enzymically synthesized FAD. The eluate was evaporated to dryness and the dry residue hydrolysed with 1 ml. of 6 N-hydrochloric acid for 2 hr. at 120°, in a sealed tube. Hydrochloric acid was removed *in vacuo* and the neutral extract was subjected to circular-paper chromatography with butanol–acetic acid–water (4:1:5, by vol.); the air-dried chromatograms were sprayed with Folin's reagent and the characteristic pink band of 4-amino-5-imidazole carboxamidine was identified with reference to a standard. Since 4-amino-5-imidazole carboxamidine is a degradation product of adenine and adenine-containing compounds this test proved the presence of an adenine moiety in the flavin in the eluate. The reaction product was hydrolysed by the crude

Table 1. *Progress of purification of flavinadenine dinucleotide-synthesizing enzyme from* Phaseolus radiatus

For details of purification, see text.

Stage of purification	Volume (ml.)	Total activity (units)	Total protein (mg.)	Specific activity (units/mg. of protein)
I	165	4062	14 930	0·272
II	180	3868	4 493	0·861
III	40	3499	483	7·232
IV	35	1928	114	14·426
V	22	1069	47	22·580

extracts of *P. radiatus* and FMN detected on chromatograms. The FAD synthesized was further identified by the D-amino acid-oxidase test (Huennekens & Felton, 1957). The substrate used was DL-alanine. There was no uptake of oxygen by the apoenzyme and the uptake of oxygen was linear with graded amounts of enzymically synthesized FAD and also with standard FAD. These tests conclusively proved the identity of the reaction product to be FAD.

Stoicheiometry of the reaction. The stoicheiometry of the FAD synthesis by the plant enzyme was established by estimating FMN utilized and FAD synthesized at different time intervals.

From Table 2 it is evident that a stoicheiometric relationship exists between FMN used and FAD synthesized.

Progress curve of the reaction. The activity at different intervals of time was determined by incubating separate sets of reaction mixtures for the time intervals mentioned in Fig. 1. There is a linearity between FAD synthesized and time up to 60 min.

pH–*Activity curves.* The effect of pH on FAD synthesis was determined by carrying out the reactions in phthalate–HCl buffer (pH 4·0–6·0; 0·1 M), veronal–HCl buffer (pH 6·0–9·0; 0·1 M) and glycine–NaOH buffer (pH 9·0–10·0; 0·1 M). The results are presented in Fig. 2.

The enzyme has a pH optimum at 7·5 in 0·1 M-veronal–HCl buffer. The following buffers were tried at the optimum pH for the effect on FAD

Table 2. *Stoicheiometry of synthesis of flavinadenine dinucleotide*

Reaction mixtures (see text) were incubated, for the time intervals mentioned, at 37°.

Time (min.)	FMN used (μm-moles/ml.)	FAD synthesized (μm-moles/ml.)
10	16·58	16·26
20	39·23	38·50
30	54·01	52·17
60	84·10	83·21
180	92·34	91·82

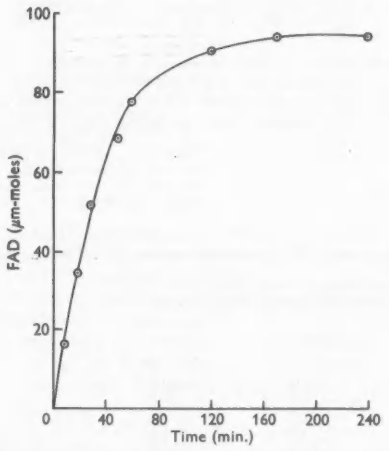


Fig. 1. Progress curve. Reaction mixtures consisted of FMN, 1 mM; ATP, 20 mM; NaF, 0·1 M; $MgSO_4$, 0·1 mM; veronal–HCl buffer (pH 7·4), 0·1 M; 0·8 ml. of enzyme preparation; total volume, 2 ml. Incubation was at 37° for the time intervals mentioned in the figure.

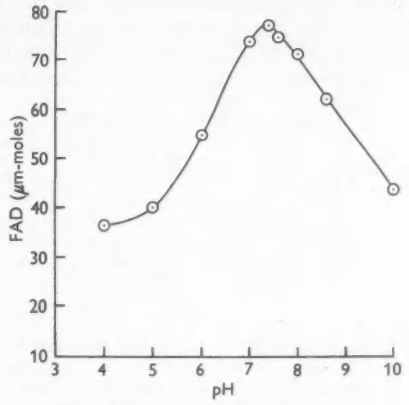


Fig. 2. pH–Activity curve. Reaction mixtures were the same as for Fig. 1 except that various buffers were used. Incubation was at 37° for 60 min.

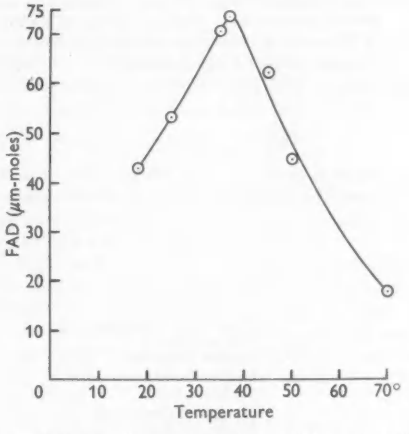


Fig. 3. Optimum temperature for activity. Reaction mixtures were the same as for Fig. 1, incubated at different temperatures for 60 min.

synthesis: 2-amino-2-hydroxymethylpropane-1:3-diol, sodium phosphate and veronal–HCl, and it was found that veronal–HCl buffer was the best.

Optimum temperature for activity. Variation of the initial rate of reaction as a function of temperature was determined by incubating the reaction mixtures at different temperatures (Fig. 3). The enzyme functions optimally at a temperature of 37°.

Substrate affinity of the enzyme. The Lineweaver–Burk plots for FMN and ATP are shown in Fig. 4. At a constant ATP concentration of 20 mM, varying concentrations of FMN were used in the reaction mixture. The enzyme functions optimally at an FMN concentration of 0·15 mM and the K_m calculated from Fig. 4 is 0·043 mM. At a constant FMN concentration of 0·20 mM varying concentrations of ATP were used in the reaction mixture. Maximum FAD synthesis was observed at an ATP concentration of 2·25 mM and K_m calculated to be 0·75 mM.

Substrate specificity. At the same molar concentration, ADP and adenosine monophosphate could not replace ATP. Riboflavin, lumiflavin or lumichrome could not replace FMN, at the same molar concentration.

Effect of ions. The various ions listed in Table 3 were tried for their effect on the FAD-synthesizing enzyme. Of the ions used, Mg^{2+} and Zn^{2+} activated at all of the three concentrations, whereas Mn^{2+} ions activated at the lowest but had no effect at mM concentration and inhibited at the highest concentration. Ni^{2+}, Cu^{2+}, Hg^{2+} and CN^- ions in-

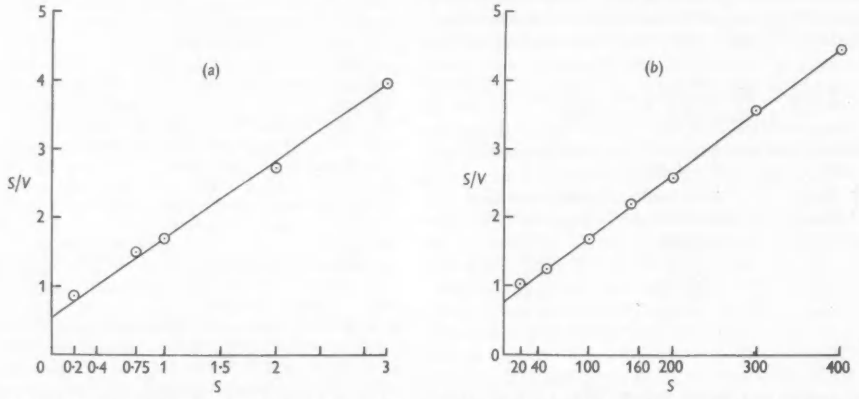


Fig. 4. Lineweaver–Burk plots. (*a*) Reaction mixtures contained 0·2 ml. of FMN, at concentrations indicated; other constituents were as given for Fig. 1; total volume, 2 ml. Incubation was at 37° for 60 min. *S*, Concn. of FMN (μM); *V*, μm-moles of FAD synthesized. (*b*) Reaction mixtures contained 0·1 ml. of ATP, at concentrations indicated; other constituents were as given for Fig. 1; total volume, 2 ml. Incubation was at 37° for 60 min. *S*, Concn. of ATP (mM); *V*, μm-moles of FAD synthesized.

Table 3. *Effect of ions on the flavinadenine dinucleotide-synthesizing enzyme*

Reaction mixtures were as follows: FMN, 1 mM; ATP, 20 mM; NaF, 0·1 M; ions at the concentrations indicated; veronal–HCl buffer (pH 7·4), 0·1 M; 0·8 ml. of enzyme; total volume, 2 ml. Incubation was at 37° for 60 min.

Substance	Percentage inhibition (−) or activation (+) at different concentrations of ions added		
	Concn. 0·1 mM	Concn. 1·0 mM	Concn. 10·0 mM
Magnesium sulphate	+12·5	+29·2	+49·2
Zinc sulphate	+4·2	+20·8	+29·4
Copper sulphate	−20·8	−16·7	−4·2
Cobalt acetate	−4·2	−4·1	−4·1
Nickel chloride	−20·8	−20·8	−12·5
Manganous sulphate	+12·5	+4·1	−20·8
Ferric sulphate	−7·5	−4·1	−4·2
Mercuric chloride	−29·1	−39·2	−40·7
Cadmium sulphate	+4·2	+4·6	+4·7
Potassium cyanide	−45·8	−50·8	−60·2

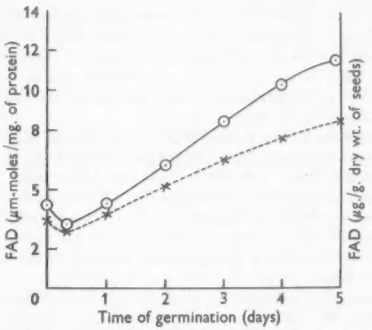


Fig. 5. Effect of germination on FAD-synthesizing activity (top curve) and FAD content of green gram (*Phaseolus radiatus*) (bottom curve). Reaction mixtures were the same as those given for Fig. 1, and were incubated at 37° for 60 min.

Table 4. *Occurrence of flavinadenine dinucleotide-synthesizing activity in plants*

Reaction mixtures were as follows: 0·2 ml. of FMN, 1 mM; 0·1 ml. of ATP, 20 mM; 0·1 ml. of NaF, 0·1 M; 0·1 ml. of $MgSO_4$, 0·1 mM; 0·7 ml. of veronal–HCl buffer (pH 7·4), 0·1 M; 0·8 ml. of enzyme from stage II; total volume, 2 ml. Incubation was at 37° for 5 hr. Sources 1–12 were resting seeds.

Source	Activity (μm-moles of FAD synthesized/ml. at 37° for 5 hr.)
1. *Cicer arietinum* (Bengal gram)	60·3
2. *Ricinus communis* (castor)	72·1
3. *Brassica campesteris* (mustard)	72·1
4. *Phaseolus mungo* (black gram)	76·7
5. *Phaseolus radiatus* (green gram)	92·1
6. *Dolichos biflorus* (horse gram)	82·1
7. *Dolichos lablab* (field bean)	93·8
8. *Cannavalia gladiata* (sword bean)	76·2
9. *Pisum sativum* (peas)	82·1
10. *Sesamum indicum* (sesame)	82·1
11. *Cajanus indicus* (red gram)	76·1
12. *Arachis hypogea* (groundnut)	60·3
13. *Solanum tuberosum* (potato)	58·1
14. *Ipomea batatus* (sweet potato)	52·7

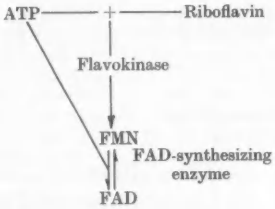
hibited at all the concentrations, whereas Co^{2+}, Fe^{3+} and Cd^{2+} ions had no effect.

Effect of germination on flavinadenine dinucleotide-synthesizing enzyme activity. In view of the observation that riboflavin content of the seedlings increases many-fold during germination (Burkholder & McViegh, 1942; Simpson, Chow & Soh, 1953; Giri *et al.* 1958), seeds were allowed to germinate in the dark, in Petri dishes. They were placed on moist cotton covered with filter-paper disks cut to the size of the Petri dishes. At definite intervals of time they were taken out, homogenized and the supernatants obtained on centrifuging at 1000 *g* for 10 min. were dialysed for 18 hr. in the cold. The supernatant obtained on centrifuging the dialysed extract at 3000 *g* for 5 min. was used as the enzyme. FAD content was determined on a parallel set of seedlings. The increase in FAD concentration paralleled the enzyme activity during germination for 6 days (Fig. 5).

Distribution of flavinadenine dinucleotide-synthesizing enzyme in plants. Fourteen plant materials were examined for this enzyme. All the plant sources investigated showed the presence of this enzyme activity (Table 4).

DISCUSSION

In the light of the experimental evidence presented in this paper the following scheme for the biosynthesis of FAD in plants appears probable:



Starting with riboflavin, FMN is synthesized by means of an irreversible reaction with ATP (Giri *et al.* 1958). The FMN so synthesized is converted into FAD by a reversible reaction with ATP.

SUMMARY

1. The occurrence of flavinadenine dinucleotide-synthesizing enzyme has been demonstrated in plants and its properties have been studied by the application of circular-paper chromatography. Fourteen plants were examined and the detailed study was confined to one of them, *Phaseolus radiatus*.

2. Under the experimental conditions described, maximum synthesis of flavinadenine dinucleotide was observed at pH 7·4. The Michaelis constant for flavin mononucleotide was 0·043 mM and adenosine triphosphate was 0·75 mM.

3. Mg^{2+} and Zn^{2+} ions activated at all the concentrations studied, whereas Mn^{2+} ions activated at the lowest concentration, but had no effect at mM and inhibited at the highest concentration. Ni^{2+}, Cu^{2+}, Hg^{2+} and CN^- ions inhibited at all the concentrations, whereas Co^{2+}, Fe^{3+} and Cd^{2+} ions had no effect.

4. An 83-fold purification of the enzyme was achieved by dialysis, fractionation with ammonium sulphate, adsorption and elution from alumina Cγ-gel, and precipitation with ethanol.

5. There was a parallel increase in flavinadenine dinucleotide content and synthesizing activity for this compound with the progress of germination.

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REFERENCES

Bessey, O. A., Lowry, O. H. & Love, R. H. (1949). *J. biol. Chem.* **180**, 755.
Bonner, J. (1942). *Bot. Gaz.* **103**, 581.
Bonner, J. & Bonner, H. (1948). *Vitam. Horm.* **6**, 225.
Burkholder, P. R. & McViegh, I. (1942). *Proc. nat. Acad. Sci., Wash.*, **28**, 1440.
Deluca, C. & Kaplan, N. O. (1958). *Biochim. biophys. Acta*, **30**, 6.
Giri, K. V. (1954). *Nature, Lond.*, **173**, 1194.
Giri, K. V. (1955). *J. Indian Inst. Sci.* **37**, 1.
Giri, K. V., Appaji Rao, N., Cama, H. R. & Kumar, S. A. (1959). *Indian Inst. Sci. Golden Jubilee Res. Vol.*, p. 103.
Giri, K. V. & Krishnaswamy, P. R. (1956). *J. Indian Inst. Sci.* **38**, 232.
Giri, K. V., Krishnaswamy, P. R. & Appaji Rao, N. (1958). *Biochem. J.* **70**, 66.
Huennekens, F. M. & Felton, S. P. (1957). In *Methods in Enzymology*, vol. 3, p. 955. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Ltd.
LePage, G. A. (1949). *Biochem. Prep.* **1**, 5.
Robinson, R. W. & Hogden, C. G. (1940). *J. biol. Chem.* **135**, 707.
Schrecker, A. W. & Kornberg, A. (1950). *J. biol. Chem.* **182**, 795.
Simpson, I. H., Chow, A. Y. & Soh, C. C. (1953). *Cereal Chem.* **30**, 222.
Trufanov, A. V. (1941). *Biokhimiya*, **6**, 301. Quoted in *Chem. Abstr.* (1941), **35**, 7499.
Watson, S. A. & Nogelle, G. R. (1947). *Plant Physiol.* **22**, 228.
Willstätter, R. & Kraut, K. (1923). *Ber. dtsch. chem. Ges.* **56**, 1117.

Biochem. J. (1960) **75**, 386

The Effect of Alcohols on the Synthesis of Lipase, Lecithinase and other Enzymes by *Bacillus cereus*

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It was found previously that the presence of any one of several lower alcohols in the growth medium inhibited the synthesis of lecithinase by growing *Bacillus cereus* (Kushner, 1957*a*). A similar effect was observed for lecithinase production in *Clostridium perfringens* by Jayko & Lichstein (1958). In both these studies the alcohols, in the concentrations used, either did not inhibit bacterial growth or had a much greater inhibitory effect on lecithinase formation than upon growth. Thus they were clearly not acting as general toxic agents or as general inhibitors of enzyme synthesis.

Their differential effect suggested that alcohols might prove valuable in other investigations of enzyme formation, and their action has been studied further. The effect of alcohols on lecithinase synthesis was first demonstrated with standing cultures of *Bacillus cereus* (Kushner, 1957*a*), in which both growth and lecithinase synthesis were suboptimum. This work has now been repeated in vigorously growing aerated cultures. The extent of utilization by the growing bacteria of the added alcohols has been determined. Finally, in order to assess the specificity of action of alcohols their effect on the formation of enzymes other than lecithinase in *Bacillus cereus* has been investigated. The enzymes studied include penicillinase, which, like lecithinase, is an extracellular enzyme, and the cell-bound enzymes phosphatase, catalase and lipase. The last-mentioned enzyme, whose synthesis was studied in some detail, was chosen because, like lecithinase, it also possesses a fatty substrate.

A preliminary account of these results has been given (Kushner, 1959).

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MATERIALS AND METHODS

Bacteria. Bacillus cereus strain 569, which has been extensively used in experiments on penicillinase formation (Pollock, 1953), was used for most of this study. For certain experiments *B. cereus* strain Mu-3055, in which the inhibitory effect of alcohols on lecithinase synthesis was first demonstrated (Kushner, 1957*a*), and the penicillinase-constitutive mutant strain 569/H (Kogut, Pollock & Tridgell, 1956), were used.

Growth medium. S broth, made as described by Pollock & Perret (1953) except that the initial steaming before autoclaving was omitted, was used for all experiments with

B. cereus strains 569 and 569/H. In most experiments 0·1 ml. of a spore suspension (containing 3×10^8 spores/ml.) was added to 10 ml. of S broth and incubated with shaking for 3–6 hr. The resulting suspension of bacteria was used as an inoculum, and amounts equivalent to 0·05 mg. of bacteria (about 3×10^7 viable cells) were added to 50 ml. of S broth in 500 ml. Erlenmeyer flasks (in some experiments proportionately smaller amounts of inoculum were added to 25 ml. or 10 ml. of broth in 250 ml. or 100 ml. flasks). For certain specified experiments the spore suspension (usually 0·05 ml./10 ml. of broth) was used as the sole inoculum. Alcohols had closely comparable effects on growth and on enzyme synthesis whether the inoculum consisted of spores or of vegetative cells. After inoculation, the flasks were incubated at 35° on a reciprocal shaker at 88 cyc./min. with a lateral throw of 3 in.

For the experiment with *B. cereus* strain Mu-3055 reported in Table 2, shaking (200 cyc./min.) and standing incubations were carried out as previously reported (Kushner, 1957*a*; Kushner & Lisson, 1959).

For lipase, phosphatase and catalase assays, the bacteria were harvested by centrifuging, washed once or twice with water and resuspended in a small volume of water, usually to a density of 10 mg. dry bacterial wt./ml. For penicillinase assays the whole bacterial culture was diluted tenfold with 0·83 mM-8-hydroxyquinoline (oxine) in 1% gelatin, at the desired time, to halt growth and enzyme synthesis. A portion (1 ml.) of this, or of a further dilution in oxine–gelatin if necessary, was used for assay.

Measurements of bacterial density were made turbidimetrically, with a Hilger Spekker absorptiometer, and are expressed as mg. dry wt. of bacteria/ml.

Enzyme assays. Lipase assays were carried out manometrically, essentially as described by Smith, Worrel & Swanson (1949). The main compartment of the Warburg vessel contained 2 ml. of 25 mN-$NaHCO_3$ and 0·5 ml. of bacterial suspension; the side arm contained 0·5 ml. of 0·2 M-tributyrin emulsified in 2% of gum acacia in 25 mN-$NaHCO_3$. Controls were run for each bacterial suspension with 25 mN-$NaHCO_3$ in the side arm to correct for endogenous output of CO_2, which was in some cases 20% of that observed in the presence of substrate. Addition of gum acacia alone did not raise the endogenous CO_2 output. For the gas phase $CO_2 + N_2$ (5:95) was used, and activity was measured at 38°. Measurements of CO_2 output were carried out for 30–60 min. Measurements on a suspension of bacteria grown in the absence of alcohols showed that in the range of activities studied lipase activity was proportional to bacterial concentration.

Penicillinase assays were carried out by the method of Henry & Housewright (1947) modified as previously described (Pollock, 1950), except that 20 000 units of penicillin were used as substrate.

Phosphatase activity was measured by following the hydrolysis of *p*-nitrophenyl phosphate under the following conditions: to 2 ml. of 0·01 M-phosphate buffer, pH 7·0, were added 0·2 ml. of a cell suspension and 1 ml. of *p*-nitrophenyl phosphate (1 mg./ml.). After incubation (usually for 30 min., but longer if the activity was low) at 38°, 1 ml. of M-K_2CO_3 was added, the bacteria were centrifuged down and the released *p*-nitrophenol was estimated spectrophotometrically at 420 mμ. Appropriate controls were carried out for colour in the substrate itself and for material absorbing at 420 mμ released from the bacteria.

Lecithinase assays were carried out as previously described (Kushner, 1957*b*). In this system, 1 unit of lecithinase activity is taken as equivalent to the liberation of 1 μmole of phosphorus from lecithin in 2 hr.

Catalase activity was measured spectrophotometrically by a method similar to that given by Chance & Maehly (1955). Silica cuvettes contained: water, 1·7 ml.; 0·05 M-H_2O_2, 0·3 ml.; 0·1 M-phosphate buffer, pH 7·0, 1 ml.; suspension of normal or toluene-treated bacteria (2 mg./ml.), 0·1 ml. After rapid mixing, the fall of E at 230 mμ was followed at room temperature (18–20°). The rate of decrease was found to be linear for 1–2 min., depending on activity. A cuvette containing water, buffer and bacteria but no H_2O_2 served as blank. Catalase activity is expressed as μmoles of H_2O_2 destroyed/mg. of bacteria/min.

A Unicam spectrophotometer model SP. 500 was employed for all spectrophotometric measurements.

Disruption of bacterial cells. Crushed-cell preparations for lipase assay were made with the Hughes press (Hughes, 1951), which was cooled to −35° before addition of the bacterial suspension. The crushed material was recovered quantitatively by washing the press with water and 50 mN-$NaHCO_3$, the final $NaHCO_3$ concentration being 25 mN. For determination of the amount of soluble-lipase activity, the crushed material was centrifuged for 15 min. at 14 750 *g* at 2°. A portion (1 ml.) of the supernatant was assayed manometrically, 1 ml. of 25 mN-$NaHCO_3$ and 0·5 ml. of water being added to the main vessel. The lipase activity of the total crushed material, before centrifuging, was also measured.

For phosphatase and catalase assays cells were disrupted by shaking the suspension in a stoppered tube with 0·1 vol. of toluene for 30 min. at 35°. In most cases, air was bubbled through the aqueous part of the suspension to remove excess of toluene before assay; it was found, however, that a small amount of remaining toluene did not affect the catalase assay.

Alcohol determination. All alcohols were measured by a variation of the method for ethanol described by Snell & Snell (1937). To a tube containing up to 0·15 m-mole of each alcohol in 1 ml. of water was added 5 ml. of 0·6% $K_2Cr_2O_7$ in conc. HNO_3–water (1:2, v/v). After heating for 2 hr. at 50° the tubes were cooled and E values at 630 mμ read against a blank prepared in the same way but containing no alcohol. Standard curves for each alcohol are given in Fig. 1. Alcohols could be measured directly in the

Fig. 1. Standard curves for alcohols. ●, Methanol; ■, ethanol; △, propan-1-ol; ○, butan-1-ol.

culture medium without prior distillation. Both S broth and Nutrient Broth (Difco) without added alcohols gave readings corresponding to a concentration of about 0·04 M-methanol; these figures were reduced by 25–50% after bacterial growth. Recoveries of alcohols added to the growth media were quantitative. All alcohol analyses were corrected for substances present in the growth medium.

Chemical compounds. The methanol and ethanol used in most experiments had been dried and redistilled before use over sodium and dimethyl or diethyl phthalate respectively; commercial material gave the same results as the redistilled alcohols. Propan-1-ol, butan-1-ol and tributyrin were British Drug House Ltd. AnalaR grade, hydrogen peroxide was Hopkins and Williams Ltd. AnalaR grade. Gum acacia was supplied by Allen and Hanburys Ltd., London. *p*-Nitrophenyl phosphate was supplied by the Sigma Chemical Co., St Louis, Mo., U.S.A.

RESULTS

Effect of alcohols on lecithinase synthesis in Bacillus cereus *strain* 569. In the earlier work on the effect of alcohols on lecithinase synthesis, *B. cereus* strain Mu-3055 was used, and growth was in standing tubes instead of shaken flasks (Kushner, 1957*a*). In shaken cultures, overnight growth was increased about tenfold and final lecithinase activity about threefold. The effects of alcohols on shaken cultures of strain 569 were closely comparable with those found in standing cultures of strain Mu-3055 (Kushner, 1957*a*) (Table 1). With shaken cultures of strain Mu-3055 similar results were also obtained.

Fig. 2 shows the relation between growth and lecithinase production in a shaken *B. cereus* culture, as well as the effect of ethanol on both processes. There was a lag in lecithinase production in relation to growth. Enzyme activity appeared only after the end of the logarithmic-growth phase, although at a time when the bacteria were still growing rapidly. A more detailed study of this relation, as well as of the relation between cell-bound and extracellular lecithinase, is to be published separately. It is seen that in this experiment ethanol inhibited growth slightly and, as expected, lecithinase production completely.

Degree of alcohol destruction during bacterial growth. Experiments carried out with both *B. cereus* strain Mu-3055 and strain 569 showed that there was little loss of added alcohols during bacterial growth (Table 2). The loss that did occur was no greater in shaken than in standing cultures of strain Mu-3055, despite the greater growth in the former. In addition, the greater part of the alcohol loss in shaken cultures of strain 569 occurred also in media with no bacteria present, presumably through evaporation.

The bacteria, then, caused little destruction of alcohols during growth, and it seemed likely that alcohols acted as such rather than as their metabolic derivatives. If this were so, then in the relatively high concentrations used the alcohols might be expected to act on the cells as lipid solvents, and a few experiments were carried out to test this possibility. A suspension of *B. cereus* strain 569 (20 mg./ml.) incubated with shaking at 35° released 1 mg. of soluble material/ml. in 2 hr. This contained 45 μg. of phosphorus, all as inorganic phosphate. Ethanol (0·35 M), propan-1-ol (0·13 M) and butan-1-ol (0·05 M) increased the phosphate (and total phosphorus) released by 10%; methanol (0·5 M) had no effect. The alcohols caused no increase in the total soluble material released, nor in the material absorbing at 260 mμ. There was no ether-soluble material (measured by dry weight and by phosphorus analysis) in intact bacterial cells treated, or untreated, with alcohols. These experiments indicated that, in the concentrations

Table 1. *Effect of alcohols on lecithinase synthesis in* Bacillus cereus *strain* 569

S broth (50 ml.) in a 250 ml. flask, with additions as shown, was inoculated with 0·1 ml. of a spore suspension and incubated with shaking for 13 hr. Lecithinase activity was measured in 0·5 ml. of the culture supernatant. In this experiment, 0·1 unit of penicillin/ml. had been added to the culture medium. Similar results were obtained in the absence of penicillin.

Alcohol added and concn. (M)	Growth (mg./ml.)	Lecithinase activity (units/mg.)
Nil	2·12	4·5
Methanol (0·5)	1·85	2·3
Ethanol (0·35)	1·62	0·5
Propan-1-ol (0·13)	1·32	0·5
Butan-1-ol (0·054)	1·92	0·5

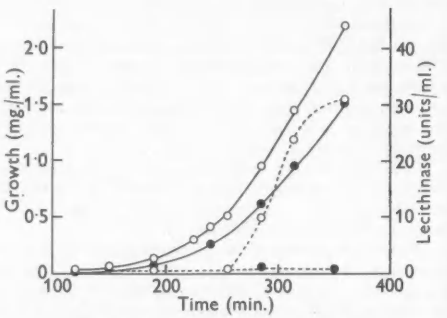

Fig. 2. Lecithinase production by growing *Bacillus cereus*; the effect of ethanol on growth and enzyme formation. Broth (50 ml.) was inoculated with 0·1 ml. of spore suspension and lecithinase activity of the whole culture determined at the times shown. Continuous lines represent bacterial growth; broken lines represent lecithinase activity. ○, Control; ●, 0·35 M-ethanol.

used, alcohols had no measurable solvent action on intact bacterial cells.

Specificity of alcohol action on enzyme formation: lipase formation. Washed cells from overnight cultures of *B. cereus* strain 569 possessed a measurable lipase activity. No activity could be detected in the external medium, and the enzyme thus appears to be wholly cell-bound. If alcohols were added to the growth medium the intracellular-lipase activity was markedly lowered, though not altogether abolished (Table 3). In the concentrations used, the alcohols had, in general, little effect on total growth in overnight experiments (Table 3). Shorter experiments (see Fig. 3) showed that

Table 2. *Destruction of alcohols by growing* Bacillus cereus

Strains Mu-3055 and 569 were grown for 14 and 17 hr. respectively. Final bacterial density (in absence of alcohol): strain Mu-3055, standing, 0·06 mg./ml., shaken, 0·6 mg./ml.; strain 569, 3·5 mg./ml. Effects of alcohols on growth of shaken cultures were as shown in later tables. Growth of standing cultures of Mu-3055 was not affected by alcohols. Mu-3055 cultures were shaken (200 cyc./min.) in 10 ml. of Nutrient Broth (Difco) in 1 in. × 8 in. tubes as described by Kushner & Lisson (1959). Cultures of 569 were shaken in 10 ml. of S broth as described in the Materials and Methods section.

B. cereus strain	Alcohol added and concn. (M)	Percentage remaining after growth: Standing culture	Percentage remaining after growth: Shaken culture
Mu-3055	Methanol (0·5)	93	94
	Ethanol (0·35)	88	87
	Propan-1-ol (0·133)	83	77
	Butan-1-ol (0·054)	80	88
		Shaken culture	Shaken medium without bacteria
569	Methanol (0·8)	88	89
	Ethanol (0·2)	77	86
	Propan-1-ol (0·08)	62	80
	Butan-1-ol (0·08)	64	75

Table 3. *Growth of* Bacillus cereus *strain 569 in the presence of alcohols, and the lipase activity of intact cells*

Bacteria were grown in 50 ml. of S broth as described in the Materials and Methods section. Figures in parentheses represent the control lipase activity (in μl. of CO_2/mg. of bacteria/hr.) in each experiment.

Expt. no.	Alcohol added	Concn. (M)	Time of incubation (hr.)	Bacterial growth (mg./ml.)	Percentage of control lipase activity
1	Nil		16	4·24	100 (66·5)
	Methanol	0·4		3·34	75
		0·8		2·70	48
	Ethanol	0·1		4·40	33
		0·2		3·96	25
		0·4		3·60	21
		0·8		3·04	30
2	Nil		17	3·50	100 (53·2)
	Methanol	0·8		2·32	64
		1·2		2·80	35
		1·6		3·10	30
3	Nil		17	4·44	100 (41·3)
	Ethanol	0·02		5·10	82
		0·04		5·50	52
		0·1		5·50	55
4	Nil		16	4·64	100 (40·6)
	Propan-1-ol	0·04		4·64	58
		0·08		3·88	46
		0·12		3·94	40
	Butan-1-ol	0·02		4·44	88
		0·04		4·64	86
		0·08		3·88	64

propan-1-ol increased the lag period and decreased the growth rate, and a similar effect was sometimes found with butan-1-ol (e.g. Table 5). Higher concentrations of propan-1-ol and butan-1-ol than those shown tended to inhibit growth completely.

Regardless of their effect on growth, the presence of each of these alcohols in the growth medium led to a lowered lipase activity/mg. of bacteria, in the washed cells. The most active alcohols in this respect (on a molar basis) were ethanol and propan-1-ol. The maximal effect of methanol was almost given by 1·2M concentration; ethanol had its maximal effect at 0·1–0·2M concentration. Propan-1-ol appeared to approach a similar maximal effect at 0·12M, the highest concentration it was possible to test. Because of its growth-inhibitory effects the maximal concentration for butan-1-ol could not be determined.

The lowered lipase activity of intact alcohol-grown cells did not by itself indicate a lowered lipase content. The cells might have been altered in such a way as to make the intracellular enzyme less accessible to its substrate. To assess this possibility, the lipase activities of cell-free extracts of normal and alcohol-grown cells were compared. Because of the relatively small effect of butan-1-ol, only methanol, ethanol and propan-1-ol were examined in these experiments. The pattern of lowered lipase activity found in intact alcohol-grown cells was also found in their extracts (Table 4).

After crushing the cells most or all of the lipase activity in the crushed material was solubilized (i.e. remained in the supernatant after centrifuging at 14 750 *g*), but recovery of lipase activity was not quantitative. Half or more of the lipase activity was lost on crushing. Attempts to avoid this loss by disrupting the cells with toluene or with ultrasonic vibration (25 kcyc., 250w at the transducer) were unsuccessful. Both methods caused almost total inactivation of the enzyme. The loss of activity limits comparison between cell-free extracts of normal and alcohol-grown cells. From the figures obtained, however, it seems very unlikely that the alcohols acted by masking the expression of lipase in the intact cell.

Effect of alcohols on the stability of formed lipase. Since washed bacterial cells were used in the above-described assays the question of inhibitory action of alcohols on lipase activity did not arise. The possibility remained, however, that the alcohols might have destroyed the lipase activity, once formed, or have acted on the cells to cause a release of lipase. These possibilities were tested in the following manner. To a culture of *B. cereus* grown overnight in the absence of alcohol, chloramphenicol (0·1 mg./ml.) was added to prevent further growth. The culture was then divided into 10 ml. lots and shaken in 100 ml. flasks, with and without the addition of alcohols. Lipase activity of these cultures was rather unstable. In the control flasks, a 30% loss of activity was found in 6 hr. and a 70% loss in 18 hr. The loss was no greater in the presence than in the absence of alcohols (in the highest concentrations shown in Table 3). Thus the alcohol effect on the final lipase activity of intact cells was not due to enzyme destruction or release. That alcohols did not cause the release of lipase from intact cells was also indicated by the fact that the cell-free medium from alcohol-grown and from normal cultures was devoid of lipase activity.

Possibility of selection of cells with low lipase-forming ability. It seemed possible that, instead of inhibiting lipase synthesis in a homogeneous bacterial population, alcohols had selectively inhibited the growth of cells with normal lipase-forming ability and permitted the growth of cells with lower ability to synthesize lipase. Such a selection might well have taken place without any measurable effect on total bacterial growth in long-term experiments. To test this alternative explanation, bacteria grown overnight in the presence of alcohols were then reinoculated into normal S broth. The amounts of alcohol carried over in the inoculum were too low to have any effect. After

Table 4. *Lipase activity in whole cells and cell-free extracts of* Bacillus cereus *strain* 569 *grown in the presence of alcohols*

Bacteria were grown for 13½ hr. (Expt. 1) or 12 hr. (Expt. 2) in 50 ml. of S broth; 60 mg. of bacteria were crushed; 10 mg. of bacteria, or the equivalent in crushed material or supernatant (obtained after centrifuging at 14 750 *g* for 15 min.), were assayed. Lipase activity: μl. of CO_2/hr./mg. of original cells.

Expt. no.	Alcohol added and concn. (M)	Lipase activity		
		Intact cells	Crushed cells	Supernatant
1	Nil	32·6	14·3	11·1
	Methanol (0·8)	19·6	9·1	7·4
	Ethanol (0·2)	19·6	6·5	4·4
2	Nil	30·1	17·2	11·8
	Ethanol (0·2)	15·6	4·4	4·6
	Propan-1-ol (0·12)	13·0	4·4	4·6

growth for 16 hr., the cells had at least as much lipase activity as control cells cultured twice in the absence of alcohols. If one discounts the very unlikely possibility that a reverse selection of high producers of lipase occurred in the absence of alcohols these experiments indicate again that alcohols caused a true inhibition of lipase formation.

Time course of lipase production by growing bacteria and the effects of alcohols. Experiments in which the effects of alcohols on lipase synthesis were followed throughout the bacterial-growth cycle showed that lipase activity/mg. of bacteria varied greatly during growth (Fig. 3). It was lowest during the early stages of growth and the logarithmic phase and rose rapidly in the late stages of growth, continuing to increase when growth, as judged by turbidity measurements, had apparently ceased. In the earlier stages of growth the presence of alcohols did not affect the formation of lipase activity; with longer experiments the higher control activity and the inhibitory effects of alcohols were seen. Thus the alcohols seemed to act preferentially on the mechanism responsible for the greatly increased rate of lipase formation in the late and stationary phases of bacterial growth. The fact that one stage of lipase synthesis was not affected by alcohols is consistent with the observation that alcohols did not completely inhibit lipase formation (Table 3).

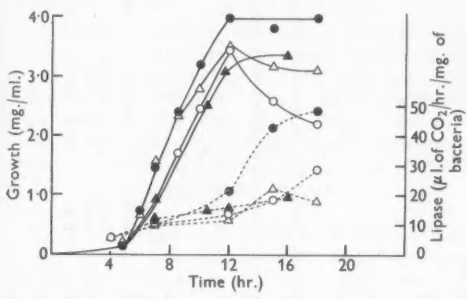

Fig. 3. Effect of alcohols on lipase formation in growing *Bacillus cereus* strain 569. S broth (50 ml.), with alcohols as shown, was inoculated with 0·25 ml. of a spore suspension (3×10^8/ml.) and samples were taken at the times shown. ●, Control flask; ○, methanol (0·8 M); △, ethanol (0·2 M); ▲, propan-1-ol (0·08 M). Continuous lines represent bacterial growth; broken lines represent lipase activity.

In the experiment shown in Fig. 3 there was a marked decrease of *E* in the methanol-grown culture, and a slight decrease in the ethanol-grown culture after 12 hr. This may have been due in part to cell lysis, but was also partly caused by a clumping of the cells, which sometimes took place in the presence of alcohols.

Crushed-cell preparations of 6 hr. cultures had approximately half the lipase activity of the intact cells, the same proportion as found in older cultures (see Table 4). This indicates that the increased lipase activity found in the older cells was not due to increased accessibility of enzyme to substrate. In the control culture, addition of chloramphenicol (0·1 mg./ml. final concentration) after growth for 12 hr. completely prevented the subsequent increase in lipase activity in the stationary culture. Thus the increased lipase activity probably involved protein synthesis.

Effect of alcohols on penicillinase and phosphatase synthesis. The effect of alcohols on penicillinase synthesis was studied both in *B. cereus* strain 569, where its formation is inducible, and in the constitutive strain 569/H (Kogut *et al.* 1956). With the inducible strain, in short-term experiments (Table 5) the alcohols increased slightly the amounts of penicillinase activity obtained after induction. In the constitutive strain there was little or no effect of alcohols on penicillinase production during 2 hr. growth in their presence. The alcohols, in the con-

Table 5. *Effect of alcohols on penicillinase synthesis*

S broth (100 ml.) was inoculated with 0·3 ml. of spore suspension (3×10^8/ml.) and shaken until a bacterial density of about 0·1 mg./ml. was reached (zero time); 10 ml. lots of the suspension were placed in 100 ml. flasks with 1 % of gelatin (final concentration) and alcohols as indicated and shaken for 2 hr. Benzylpenicillin (10 units/ml.) was added to 569 cultures at zero time and at 1 hr. Pen-ase, penicillinase (expressed as units/mg. of bacteria).

	B. cereus strain					
	569		569/H			
	Expt. 1		Expt. 2		Expt. 3	
Alcohol added and concn. (M)	Growth (mg./ml.)	Pen-ase	Growth (mg./ml.)	Pen-ase	Growth (mg./ml.)	Pen-ase
Nil	0·66	330	0·75	2175	0·85	1972
Methanol (0·8)	0·52	360	0·62	2090	0·70	1982
Ethanol (0·2)	0·56	409	0·78	2155	0·78	1962
Propan-1-ol (0·04)	0·38	412	0·38	2975	0·50	2090
Butan-1-ol (0·08)	0·24	435	0·40	2190	0·54	1832

Table 6. *Effect of alcohols on phosphatase formation in growing* Bacillus cereus *strain* 569

For Expts. 1 and 3, 100 ml. of S broth was inoculated with 0·3 ml. of a spore suspension (3×10^8/ml.) and shaken for 4 hr., then divided into 10 ml. lots in 100 ml. flasks, and alcohols were added as shown. In Expts. 2 and 4 inoculations of spores (0·05 ml./10 ml. of broth) were made into control and alcohol-containing media. Phosphatase is expressed as μmole of *p*-nitrophenol/mg. of bacteria/hr. Figures in parentheses show phosphatase activity of toluene-treated cells.

		Alcohol added and concn. (M)									
		Nil		Methanol (0·8)		Ethanol (0·2)		Propanol (0·04)		Butanol (0·04)	
Expt. no.	Time of growth (hr.)	Growth (mg./ml.)	Phosphatase	Growth (mg./ml.)	Phosphatase	Growth (mg./ml.)	Phosphatase	Growth (mg./ml.)	Phosphatase	Growth (mg./ml.)	Phosphatase
1	5	0·312	0·232	0·267	0·200	0·310	0·204	0·205	0·419	0·280	0·218
	6	0·820	0·107	0·660	0·139	0·840	0·100	0·074	0·343	0·720	0·120
2	6	1·28	0·116	0·660	0·193	1·04	0·117	0·840	0·233	0·780	0·149
	10	3·69	0·015	3·13	0·012	3·63	0·009	2·78	0·025	3·40	0·015
3	4	0·100	0·702 (0·711)	—	—	—	—	—	—	—	—
	6	0·720	0·238 (0·225)	0·620	0·209 (0·255)	0·740	0·183 (0·241)	0·110	0·440 (0·389)	0·700	0·177 (0·239)
4	16	2·5	0·021 (0·007)	—	—	—	—	—	—	—	—

Table 7. *Effect of alcohols on catalase formation in growing* Bacillus cereus *strain* 569

Conditions were as given in Table 6, Expts. 2 and 4. Catalase activity: μmoles of H_2O_2 destroyed/mg. of bacteria/min. Figures in parentheses show catalase activity of toluene-treated cells.

	Alcohol added and concn. (M)									
	Nil		Methanol (0·8)		Ethanol (0·2)		Propanol (0·08)		Butanol (0·04)	
Time of growth (hr.)	Growth (mg./ml.)	Catalase	Growth (mg./ml.)	Catalase	Growth (mg./ml.)	Catalase	Growth (mg./ml.)	Catalase	Growth (mg./ml.)	Catalase
6	1·70	3·8 (3·1)	1·12	4·2 (2·8)	1·56	4·8 (3·3)	1·14	6·3 (6·6)	1·36	3·1 (3·1)
11	3·60	3·5 (3·1)	3·70	5·6 (6·1)	4·00	4·7 (4·8)	3·30	5·0 (4·2)	3·86	3·8 (4·8)
16	3·60	8·9 (21·9)	2·20	19·0 (26·8)	3·10	10·0 (23·0)	3·90	8·0 (29·0)	2·90	10·0 (24·5)

centrations used in these assays, had no effect on penicillinase activity.

Penicillinase production could not be measured accurately after overnight incubation. The amounts of penicillinase produced per mg. of bacteria were much lower than those shown in Table 5, and the results were too erratic to interpret. The loss of activity may have been due to penicillinase destruction by proteinase action (Kogut *et al.* 1956) but was not further investigated. It was concluded from these experiments that in short-term incubations alcohols had little or no effect on penicillinase synthesis.

The time course of phosphatase synthesis and the effect of alcohols on this process are shown in Table 6. Phosphatase synthesis obviously took place very early in the growth cycle. The activity per mg. of cells decreased after the first measurement possible, and phosphatase synthesis, in terms of total activity, was ended 6 hr. after inoculation of the spores into the medium. Despite some variation between individual experiments, the alcohols, with the exception of propan-1-ol, had little effect on phosphatase synthesis; propan-1-ol appeared to stimulate this synthesis. Toluene treatment caused approximately the same small increase in the phosphatase activity of young control and alcohol-grown cells, whereas the very low activity of overnight cultures was further reduced by toluene treatment.

Effect of alcohols on catalase formation. The syntheses of lecithinase, penicillinase and phosphatase all take place relatively early in the bacterial-growth cycle. The effect of alcohols on the syntheses of these enzymes may not be directly comparable with their effect on lipase formation, a process that takes place in a later phase of growth. For this reason, catalase, an enzyme whose activity increases during growth in a manner similar to that of lipase, was of special interest in analysing the alcohol effect.

Table 7 shows the changes of catalase activity in intact and toluene-treated cells grown in the presence and in the absence of alcohols. As with lipase, catalase activity was low in the early stages of growth but increased rapidly between 11 and 16 hr., during the stationary growth phase; this late increase in catalase activity was completely prevented by 0·1 mg. of chloramphenicol/ml. Toluene treatment did not affect the activity of young cells, but increased by 100% or more the activity of 16 hr. cultures. Growth in the presence of alcohols had no, or a slightly stimulatory, effect on the catalase activity of toluene-treated cells, and, with the exception of methanol, little effect on the activity of intact cells. Intact cells grown in the presence of methanol for 16 hr. had a higher catalase activity than control cells, but toluene had less stimulatory effect on their activity. Toluene-treated control and methanol-grown cells had, finally, approximately the same activity. This suggests that growth in the presence of methanol may have had the same effect as treatment with toluene.

DISCUSSION

Except for the experiments on lecithinase formation there appear to have been no systematic studies of alcohol action on the formation of specific enzymes, and few isolated observations. Baba, Hayashi & Oshima (1955) found that there was more formation of invertase in yeast when glucose was used as a carbon source than when ethanol was used. Satomura, Oi & Sawada (1958) observed recently that in the fungus *Sclerotinia libertiana* ethanol, together with a number of sugars, inhibited lipase formation. Ethanol enhanced mycelial growth and was thought to be utilized by the organism. If so, the action of ethanol on enzyme synthesis in both the above-mentioned organisms may have been fundamentally different from that found on lecithinase and lipase synthesis, since alcohols did not appear to be used as substrates for growth by the *B. cereus* strains studied.

To date, lecithinase formation in two bacterial species and lipase formation in one have been found to be inhibited by alcohols. The present survey has shown that penicillinase, phosphatase and catalase formations in *B. cereus* were not inhibited; nor, presumably, was the formation of many other enzymes since the bacteria grew in the presence of alcohols. One can obviously not draw a general rule of alcohol action from the few enzymes studied, but it is curious and may be significant that thus far the enzymes whose production has been found to be inhibited by alcohols are those concerned with the degradation of fatty substrates.

The fact that alcohols inhibit both lipase and lecithinase formation does not necessarily mean that the basis of action in each case is the same. One striking difference is that with lecithinase formation the inhibitory power of alcohols increases with increasing molecular weight (Kushner, 1957*a*), whereas with lipase formation butanol is less effective than propanol or even ethanol. A more important difference may lie in the fact that alcohols can inhibit lecithinase formation completely, whereas in the early stages of growth lipase formation is unaffected by alcohols.

The fact that most of the lipase activity appears late in the growth cycle and that alcohols appear to act specifically on this late formation makes comparison between lipase and other enzymes difficult.

During the period when alcohols inhibited lipase formation, lecithinase and phosphatase were no longer being formed and the formation of penicillinase could not easily be studied. It might be argued that the alcohol inhibition of lipase formation was in some way connected with this enzyme's increase late in the growth cycle, were it not that the formation of catalase, most of which also takes place late in the growth cycle, was not inhibited by alcohols.

It was, as stated, the apparent similarity between the substrates of lipase and lecithinase that led to the inclusion of lipase in this study. The substrate specificity of *B. cereus* lipase, however, has not yet been studied in any detail. Intact cells, which could hydrolyse tributyrin, were ten times as active against triacetin, both in solution (40mM) and as an emulsion (0·8M). They did not hydrolyse ethyl acetate (2 mM) or soluble ethyl butyrate (33 mM) but acted on an ethyl butyrate emulsion (0·45M). If the same enzyme were acting on all substrates then in some, though not all, of its properties *B. cereus* lipase resembles the pancreatic lipase described recently by Sarda & Desnuelle (1958), which does not hydrolyse water-soluble substrates but apparently requires an oil–water interface for its action.

Two possible mechanisms of alcohol action have been considered. By the first, both lecithinase and lipase synthesis would depend on the presence in the cell of an internal inducer, either phospholipid or triglyceride in nature. Although the concentrations of alcohols used were not high enough to extract lipids from the bacterial cell, it might be possible that the presence of alcohols prevented fatty inducers from making effective contact with the appropriate enzyme-forming systems.

As an alternative, the necessity for an enzyme acting on a fatty substrate to overcome a phase barrier may provide a clue to the mechanism of alcohol action. Much attention has been given recently to the special conditions necessary for enzyme action on fatty substrates. In addition to pancreatic lipase one can cite the phospholipase B of *Penicillium notatum*, which requires the presence of certain lipids in order to attack lecithin (Bangham & Dawson, 1959; Dawson & Bangham, 1959), the role of the lipids being to introduce negative groups essential for enzyme action on the surface of the lecithin particle. Phospholipases of different types and from various sources have been found to be stimulated by the presence of ether and other organic solvents (Hanahan, 1952; Kates, 1957; Hanahan & Vercamer, 1954; Kushner, 1957*b*), these being necessary for contact between enzyme and substrate.

By the second hypothesis, alcohols would not necessarily inhibit the total synthesis of an enzyme protein. Instead, it may be that the active enzyme contains as an essential part a group, possibly a lipid group, enabling it to come into effective contact with its phospholipid or triglyceride substrate, and that during growth in the presence of relatively high concentrations of alcohols attachment of this group to the inactive protein does not take place.

SUMMARY

1. The inhibitory effects of lower alcohols on lecithinase synthesis, first observed in standing cultures of *Bacillus cereus* strain Mu-3055, have now been found in shaken, vigorously growing cultures of this organism and of *B. cereus* strain 569.

2. During growth in the presence of alcohols both *B. cereus* strain 569 and *B. cereus* strain Mu-3055 caused little alcohol destruction. Alcohols, in the concentrations used in these experiments, had little or no detectable chemical action on intact bacterial cells.

3. Intact cells and cell-free extracts of *B. cereus* strain 569 grown in the presence of each of four lower alcohols had less lipase activity than cells or extracts of cells grown in the absence of alcohols. Ethanol and propan-1-ol were more active in this respect than methanol or butan-1-ol. The alcohols had no effect on the stability of lipase, once formed. They did not selectively promote the growth of cell lines with a lowered lipase activity. Their inhibitory effect appeared to be on the formation of the active enzyme.

4. The lipase content of *B. cereus* strain 569 varied greatly during bacterial growth. It was lowest in the logarithmic phase of growth and increased after the end of this phase; the greatest increase in lipase activity/mg. of bacteria took place during the stationary growth phase. Alcohols did not inhibit the early formation of lipase activity, but appeared to act specifically on that which took place in the late growth phases.

5. Penicillinase formation, both induced (in *B. cereus* strain 569) and constitutive (in *B. cereus* strain 569/H), was not inhibited by alcohols during and immediately after the logarithmic phase of growth.

6. Phosphatase formation took place early in the bacterial-growth cycle. With the exception of propan-1-ol, which had a stimulatory action, the alcohols were without effect on phosphatase formation.

7. The catalase activity of *B. cereus* strain 569, which increased late in growth in a manner similar to lipase, was not inhibited by alcohols.

8. So far, alcohols have been found to inhibit the formation of only those enzymes concerned with the degradation of fatty substrates. Possible mechanisms of alcohol action are discussed.

I am greatly indebted to Dr M. R. Pollock for providing the facilities for this work and to him and Dr H. J. Rogers for many helpful discussions while this work was going on. Miss Ruth Coyle provided expert and invaluable technical assistance. This paper is contribution no. 583, Forest Biology Division, Research Branch, Canada Department of Agriculture, Ottawa, Ontario, Canada. This study was carried out under a work transfer from the Forest Insect Laboratory, Sault Ste. Marie, Ontario, Canada.

REFERENCES

Baba, T., Hayashi, M. & Oshima, M. (1955). *Bull. Fac. Eng., Hiroshima Univ.* **4**, 287. Quoted in *Chem. Abstr.* (1956), **50**, 5831g.

Bangham, A. D. & Dawson, R. M. C. (1959). *Biochem. J.* **72**, 486.

Chance, B. & Maehly, A. C. (1955). In *Methods in Enzymology*, vol. 2, p. 764. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Dawson, R. M. C. & Bangham, A. D. (1959). *Biochem. J.* **72**, 493.

Hanahan, D. J. (1952). *J. biol. Chem.* **195**, 199.

Hanahan, D. J. & Vercamer, R. (1954). *J. Amer. chem. Soc.* **76**, 1804.

Henry, R. J. & Housewright, R. D. (1947). *J. biol. Chem.* **167**, 559.

Hughes, D. E. (1951). *Brit. J. exp. Path.* **32**, 97.

Jayko, L. G. & Lichstein, H. C. (1958). *Proc. Soc. exp. Biol., N.Y.*, **98**, 63.

Kates, M. (1957). *Canad. J. Biochem. Physiol.* **35**, 127.

Kogut, M., Pollock, M. R. & Tridgell, E. J. (1956). *Biochem. J.* **62**, 391.

Kushner, D. J. (1957*a*). *Nature, Lond.*, **179**, 781.

Kushner, D. J. (1957*b*). *J. Bact.* **73**, 297.

Kushner, D. J. (1959). *Biochem. J.* **73**, 17P.

Kushner, D. J. & Lisson, T. A. (1959). *J. gen. Microbiol.* **21**, 96.

Pollock, M. R. (1950). *Brit. J. exp. Path.* **31**, 739.

Pollock, M. R. (1953). *Symp. Soc. gen. Microbiol.* **3**, 150.

Pollock, M. R. & Perret, C. J. (1953). *J. gen. Microbiol.* **8**, 186.

Sarda, L. & Desnuelle, P. (1958). *Biochim. biophys. Acta*, **30**, 513.

Satomura, Y., Oi, S. & Sawada, A. (1958). *Bull. agric. chem. Soc. Japan*, **22**, 194.

Smith, G. N., Worrel, C. S. & Swanson, A. L. (1949). *J. Bact.* **58**, 803.

Snell, F. D. & Snell, C. T. (1937). *Colorimetric Methods of Analysis*, vol. 2. London: Chapman and Hall Ltd.

Biochem. J. (1960) **75**, 395

The Biological Activity of Pure Peptides obtained by Enzymic Hydrolysis of Insulin

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Although much research has been done on the biological activity of material obtained by the action of enzymes on insulin, none of these investigations has been carried out with highly purified peptides of known structure. The determination of the structure of insulin by Sanger and his colleagues (Ryle, Sanger, Smith & Kitai, 1955) has made possible the assignment of known structures to the products of the proteolysis of insulin under controlled conditions. The present investigation was undertaken to assess the biological activity, by more than one criterion, of purified peptides obtained from insulin by the action of carboxypeptidase and trypsin.

Treatment with carboxypeptidase liberates alanine from the *C*-terminal end of the phenylalanyl chain (Fig. 1). The residual molecule (DA insulin) was reported by Lens (1949) to have only slight hypoglycaemic activity in rabbits, whereas Harris & Li (1952) found it to be fully active by the mouse-convulsion assay. The present results confirm those of Harris & Li.

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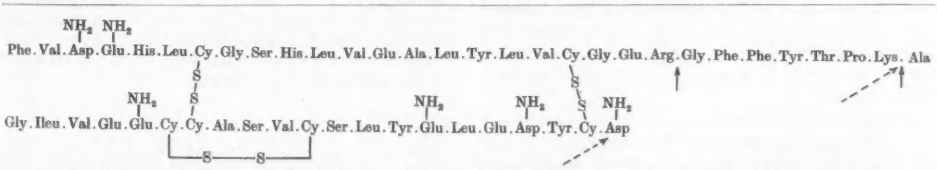

Fig. 1. Amino acid sequence in ox insulin. †, Site of action of trypsin; ↗, site of action of carboxypeptidase.

The other *C*-terminal residue of insulin on the glycyl chain (asparagine) is split off much more slowly by carboxypeptidase, giving rise to a derivative (DAA insulin) with the two *C*-terminal residues removed. Although Harris & Li reported an almost quantitative removal of the asparagine residue, leading to an inactive product, we have been unable to obtain a yield of more than 27%, which made estimation of biological activity difficult since the product could not be separated from the unchanged insulin. However, Sanger & Thompson (1953) showed that a much better yield of asparagine could be obtained from *N*-acetylinsulin and it was possible to obtain a product (DAA acetylinsulin) from which the asparagine was almost quantitatively removed. (The introduction of acetyl groups, which under their conditions had reacted only with three amino groups, does not affect the activity of the insulin.)

Since the discovery of insulin, trypsin has been the enzyme most commonly used to investigate the influence of modification of the insulin molecule on its biological activity. The impurity of the enzyme preparation led to results with insulin which were not always clear. Butler, Dodds, Phillips & Stephen (1948, 1949) showed that crystalline trypsin broke only one or two peptide bonds; although only a little non-protein nitrogen was formed there was much inactivation. This, however, was not confirmed by Van Abeele & Campbell (1951), who found no loss of activity. Harris & Li (1952) found that, under the conditions used by Van Abeele & Campbell, only alanine was removed. Two bonds, in positions 22–23 (Arg.Gly) and 29–30 (Lys.Ala) in the phenylalanyl chain, are susceptible to the action of trypsin. The latter bond is split relatively rapidly giving rise to free alanine and DA insulin, the same products as those obtained with carboxypeptidase. Although the Arg.Gly bond is rapidly split in the isolated B chain from oxidized insulin, it goes relatively slowly with intact insulin, giving rise to free alanine, a heptapeptide (Gly.Phe.Phe.Tyr.Thr.Pro.Lys) and the remainder of the molecule (DHA insulin). Various conditions of hydrolysis with trypsin were studied in an attempt to improve the yield of DHA insulin and the best yield (39%) was obtained with insulin from which the zinc had been removed, as had been found with leucine amino peptidase (E. L. Smith, personal communication). With a partition column (Andersen, 1954; Nicol & Smith, 1956) it was possible to separate the DHA insulin from unchanged insulin, and to obtain it in a pure form suitable for biological assay.

The above-mentioned derivatives were all tested for biological activity both by the rabbit-hypoglycaemic test and in the isolated rat diaphragm. The two methods were used to ensure the reliability of the results and also to ascertain whether their activities differed in any qualitative way from that shown by insulin.

EXPERIMENTAL

For definition of abbreviations used for amino acids in this paper see *Biochem. J.* (1957), **66**, 6.

Insulin. Insulin (Boots Pure Drug Co. Ltd.), three times recrystallized, was used. Acetylinsulin was prepared according to the method of Fraenkel-Conrat & Fraenkel-Conrat (1950).

Technical methods. Spectrophotometric measurements were made with a Unicam SP. 500 spectrophotometer. Peptide materials were dried in a desiccator over sodium hydroxide and sulphuric acid.

Action of carboxypeptidase

DA insulin. Carboxypeptidase (The Armour Laboratories; recrystallized) was treated with di*iso*propyl phosphorofluoridate (DFP) to inactivate any contaminating endopeptidases as follows.

A portion (1·45 ml.) of mM-DFP was added to 0·5 ml. of the carboxypeptidase preparation containing 0·25 mg. of the enzyme. The pH was adjusted to 7·5 and the mixture was incubated at 37° for 30 min. A control of the same quantity of DFP with 0·5 ml. of water instead of enzyme was also incubated at pH 7·5. Insulin (25 mg.) was dissolved in both enzyme and control solutions. The pH of both was adjusted to 7·8 by the addition of 0·1N-acetic acid and they were incubated at 37° for 6·5 hr.

The solution was then adjusted to pH 5·4 with 0·1N-acetic acid. This precipitated the DA insulin and enzyme protein, which were washed with water, dried in a desiccator and used for biological assay. An approximate estimate of the free amino acids in the supernatant was by the spot-dilution method (Polson, Mosley & Wyckoff, 1947) with alanine and asparagine standards. The yields were 0·9 mole of alanine and 0·1 mole of asparagine/mole of insulin.

DAA insulin. Insulin (5 mg.) and 0·5 mg. of DFP-treated carboxypeptidase were incubated under the same conditions as described above for 19·5 hr. After adjustment of the pH to 5·4, the precipitate (DAA insulin) was washed with water and dried. The supernatant was found to contain 0·72 mole of alanine and 0·27 mole of asparagine. Estimation was carried out by the preparation of the dinitrophenyl (DNP) derivatives (Levy, 1954; Blackburn & Lowther, 1950). Recovery experiments had first been done with known weights of the amino acids, resulting in 95% correct recovery.

DAA acetylinsulin. Carboxypeptidase (0·1 mg.) was incubated in 2 ml. of 2 mM-DFP at pH 7·8 for 0·5 hr. and then added to 5 mg. of acetylinsulin. After incubation at 37° for 19 hr. the DAA acetylinsulin was precipitated by adjusting the solution to pH 4·2 and was washed with water and dried. The supernatant contained approx. 1 mole of alanine and 1 mole of asparagine according to the spot-dilution method (Polson *et al.* 1947).

Action of trypsin on insulin

Insulin (10 mg.) was incubated with 1 mg. of trypsin (The Armour Laboratories; three times recrystallized) at 37° and pH 7·5 for 24 hr. Individual solutions of insulin

and trypsin were kept separately and similarly incubated as controls. The enzymic digest was brought to pH 5·2 at the end of the experiment and centrifuged. The supernatant fluid contained the heptapeptide and alanine; the precipitate, consisting of a mixture of insulin, DHA insulin and a small quantity of trypsin, was dried in a desiccator.

The supernatant fluid was then subjected to ionophoresis at pH 6·5 (pyridine–acetic acid–water buffer; 10 ml.: 0·4 ml., made up to 100 ml. with water) on Whatman no. 4 paper for 3 hr. at 37·5 v/cm. The alanine and heptapeptide bands were 3 and 12 cm. respectively from the origin on the cathode side. Their positions were recognized by developing a marker strip with ninhydrin or by very rapid examination of the whole sheet under the u.v. lamp. They were cut out, eluted and their identity was satisfactorily confirmed in the first experiment by hydrolysis and paper chromatography; in subsequent experiments the eluates of the heptapeptide were used for the estimation of the extent of the trypsin breakdown of insulin. The quantity of heptapeptide released and recovered by ionophoresis was determined either by hydrolysis and determination of the amino-nitrogen content by the ninhydrin method (Moore & Stein, 1951) or by treating the eluate with α-nitroso-β-naphthol and nitric acid and measuring $E_{520\,m\mu}$ due to tyrosine (Gerngross, Voss & Herfeld, 1933).

With normal insulin the yield of heptapeptide was about 0·16 mole/mole of insulin. No improvement was found with calcium-stabilized trypsin (Kilby & Youatt, 1954) or when DA insulin was used as substrate. However, when insulin of a low (0·025%) zinc content (Sluyterman, 1955) was used the yield of heptapeptide was 0·39 mole.

Separation of DHA insulin. Silane-treated Celite 545 (Howard & Martin, 1950) was ground with the upper phase of a butan-2-ol–0·05 M-trichloroacetic acid (1·0:2·5, v/v) equilibrated mixture. A thick slurry was made with the lower phase and a glass column (20 cm. long and 2 cm. diam.) was packed with it. The dried precipitate from the tryptic-hydrolysis experiment was dissolved in a minimum quantity of the lower phase and introduced on the column, which was developed with the lower phase at 25° for 12 hr.; 100 fractions of 1·25 ml. were collected with an automatic fraction collector. The protein concentration in each fraction was determined from $E_{275\,m\mu}$ by using a Unicam spectrophotometer SP. 500. There were two obvious peaks, the first due to DHA insulin and the second to unchanged insulin. The first peak was much larger in the experiments in which zinc-free insulin was used as a substrate. The solution containing DHA insulin was concentrated *in vacuo* at room temperature to 1–2 ml. and washed twice with ether to remove trichloroacetic acid. The suspension was redissolved in 1 drop of aq. N-NH_3 solution and brought to pH 5·2. The precipitate (DHA insulin) was centrifuged off, washed with a little water, then with a large quantity of acetone and afterwards dried *in vacuo*. It was assumed that this treatment had not affected the DHA insulin, since a control sample of insulin with low zinc content, which was passed through a similar column and extracted in the same way, did not show any loss of biological activity by the two tests when compared with an untreated sample.

The composition and integrity of DHA insulin was confirmed by the DNP end-group-assay method (Sanger, 1945) and, after hydrolysis, by two-dimensional paper chromatography in a phenol–water/butanol–acetic acid system. An amino acid analysis of DHA insulin by the Moore & Stein (1951) method carried out by Mr M. W. Rees and Miss B. Worboys of this Department gave values in agreement with those calculated for it (Table 1). A sample of insulin was analysed at the same time and gave results which agreed with its known structure (Ryle *et al.* 1955). In particular, no threonine could be detected in DHA insulin. As this amino acid occurs only in the last eight residues of the B chain, which had evidently been removed by trypsin, its absence in the analysis of DHA insulin showed that not more than 3% of the unchanged insulin could be present as a contaminant, since a higher figure would have been detected.

There was a possibility that DHA insulin might be more easily denaturable than insulin (as, for example, during the acetone extraction) and might in its pure form after tryptic digestion, be still capable of full instead of partial insulin activity. The only way in which this could be tested was to dissolve the tryptic digest of zinc-free insulin at the end of incubation, without attempting any separation, and test this directly for glucose uptake by the isolated rat diaphragm.

Zinc-free insulin (10 mg.) was dissolved in 5 ml. of 0·1 N-acetic acid and 2 mg. of trypsin added. Two digests were prepared. The first, which was the control, was brought to pH 3·2 and kept at 4°. This ensured that there was no trypsin action. The other was incubated under the usual conditions at 37°. Both were left for 24 hr. under these conditions, a trace of toluene being added to each. At the end of this period the second digest (at 37°) was brought to pH 2·5. To each, 3 mg. of soya-bean trypsin inhibitor (The Armour Laboratories) was added to prevent the trypsin

Table 1. *Amino acid analysis of DHA insulin (Moore & Stein, 1951)*

DHA insulin (5·2 mg.; 0·72 mg. of N) was used. Number of residues was calculated assuming 54 N atoms/molecule (13·3 μg. of N/residue).

Amino acid	Wt. of α-amino N (μg.)	No. of residues	No. of residues in ox insulin
Aspartic acid	38·4	2·9	3
Threonine	0	0	1
Serine	36·7	2·8	3
Proline	0	0	1
Glutamic acid	91·1	6·8	7
Glycine	39·0	2·9	4
Alanine	28·7	2·1	3
Valine	63·2	4·7	5
Half-cystine*	(40·2)	(3·0)	6
Isoleucine	10·7	0·8	1
Leucine	74·8	5·6	6
Tyrosine	38·5	2·9	4
Phenylalanine	14·6	1·1	3
Lysine	0	0	1
Histidine	24·5	1·8	2
Arginine	13·1	1·0	1
NH_3†	116·0	8·7	6

* Cystine analyses are unreliable with the method used.

† The value for ammonia N is considerably higher than that calculated for the theoretical amide content, indicating that the preparation was slightly contaminated with ammonia. This accounts for the slightly low values for most amino acids.

affecting the hemidiaphragms. The digests were then assayed for activity. Portions of solutions of the experimental and control digests which had contained 0·5 μg. of insulin before incubation were used for assay.

Biological tests

Rabbit hypoglycaemia. Two or four fasting rabbits were used for the tests and were divided into two groups. Samples of blood were taken from an ear vein every hour for 3 hr. Then an injection of the test substance was made into a vein in the other ear. Duplicate blood samples were taken when possible at 5, 20, 50, 75, 90 and 120 min. The blood samples were put into 3 ml. of water containing a little fluoride, deproteinized and the glucose content was determined (Nelson, 1944; Somogyi, 1941). The other group was treated in a similar way but in this case a known weight of insulin was injected. After 2 days the groups were reversed and the treatment was repeated. The percentage fall in concentration of blood sugar was determined. Rabbits were chosen for use when their fasting blood sugar did not vary by more than 10 mg./100 ml. Not enough rabbits were used for a full-scale assay. All that was sought was a comparison under the same conditions of the action of different doses of insulin and its derivatives on the rabbit-blood sugar.

Glucose uptake of the isolated rat hemidiaphragm. This method was carried out with Gey's bicarbonate buffer (Gey & Gey, 1936) as used by Randle (1956) and others. The absolute value of the basal uptake of glucose by the hemidiaphragm varies with different experiments. Comparisons were made with experiments done simultaneously with the buffer control, and with the test substance.

RESULTS AND DISCUSSION

C-*Terminal alanine*

The results with DA insulin are shown in Fig. 2 and confirm the findings of Harris & Li (1952) that it is fully active. The *C*-terminal alanine residue is thus not essential for biological activity.

C-*Terminal asparagine*

When 0·27 mole of asparagine had been removed the activity in the rabbit of the resulting DAA insulin was lower than that of insulin (Fig. 3) as matched in the same series of four rabbits. This again confirms the findings of Harris & Li. Clearer results were, however, obtained with the derivative of acetylinsulin (Table 2). Although acetylinsulin itself showed full activity, no activity could be detected with 150 μg. of the DAA acetylinsulin from which both alanine and asparagine had been split completely by carboxypeptidase. With 1500 μg. of DAA acetylinsulin there was some activity which was less than that of 150 μg. of acetylinsulin.

The *C*-terminal asparagine residue of the glycyl chain is thus much more important for biological activity than is the *C*-terminal alanine residue of the phenylalanyl chain.

Heptapeptide sequence

The free heptapeptide (Gly.Phe.Phe.Tyr.Thr.Pro.Lys) that is split off from insulin by the action of trypsin was found to have no effect on the rabbit-blood sugar (Table 2). With the rat diaphragm 0·125, 10 and 20 μg. gave no significant response (Table 3).

A preliminary biological test of an incompletely characterized preparation of DHA insulin revealed no hypoglycaemic activity in the rabbit (Nicol & Smith, 1956). Nevertheless, samples of the peptide obtained by evaporation *in vacuo* from the butanol

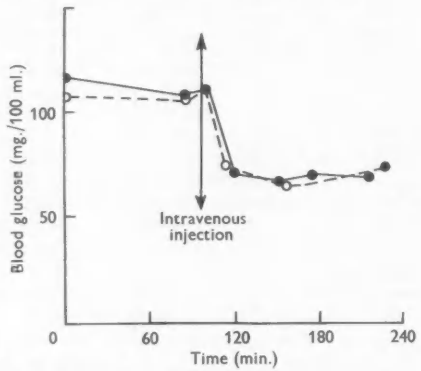

Fig. 2. Effect of insulin and carboxypeptidase-treated insulin (DA insulin) on the blood glucose of the fasting rabbit. The carboxypeptidase had removed 0·9 mole of alanine and less than 0·1 mole of asparagine/mole of insulin. ○, Insulin 150 μg.; ●, DA insulin 150 μg.

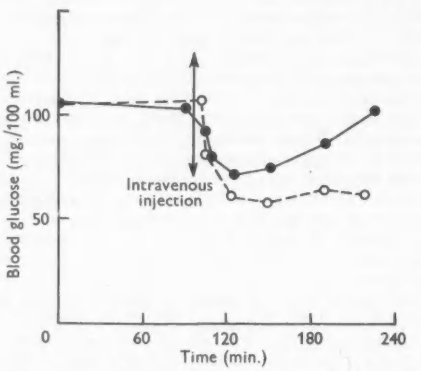

Fig. 3. Effect of insulin and carboxypeptidase-treated insulin (DAA insulin) on the blood glucose of the fasting rabbit. The carboxypeptidase had removed 0·72 mole of alanine and 0·27 mole of asparagine/mole of insulin. ○, Insulin 150 μg.; ●, DAA insulin 150 μg.

Table 2. *Maximum fall of blood glucose of fasting rabbits after intravenous injection of insulin derivatives*

Substance	No. of rabbits	Dose (μg.)	Maximum fall of blood glucose (percentage of original blood glucose)	Remarks
Insulin	8	150	55	—
Acetylinsulin	6	150	54	Full activity
DAA acetylinsulin	2	150	4	No activity
	2	1500	34	Less than 10% of original activity
DHA insulin	4	150	9	No activity
	4	1000	48	Approx. equivalent to 150 μg. of insulin
DHA insulin + Insulin	2	1000 + 200	63	Insulin activity not impaired
Heptapeptide	2	1000	8	No activity
Heptapeptide + Insulin	2	1000 + 150	56	Insulin activity not impaired

Table 3. *Effect of the heptapeptide on the glucose uptake of the isolated rat diaphragm*

Medium contained 2·5 mg. of glucose/ml. One hemidiaphragm in 1 ml. of medium was incubated for 2–3 hr. at 37° under $O_2 + CO_2$ (95:5) (Randle, 1956). Figures in parentheses show the number of hemidiaphragms used.

Amount of heptapeptide (μg.)	Uptake of glucose (mean ± S.D.; mg. of glucose/g. of tissue/hr.) Control	Experimental	P (%)
0·125	1·57 ± 0·26 (5)	1·54 ± 0·26 (5)	> 5
10	1·81 ± 0·20 (4)	2·21 ± 0·16 (6)	> 5
20	3·23 ± 0·51 (5)	3·61 ± 0·51 (5)	> 5

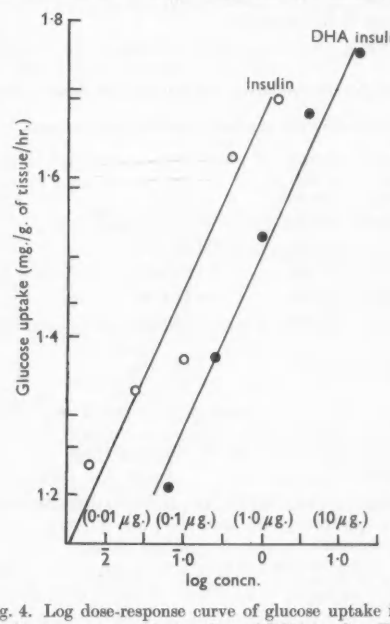

Fig. 4. Log dose-response curve of glucose uptake in the rat diaphragm caused by insulin and DHA insulin. Experimental details were as given in Table 3 (Randle, 1956). ○, Insulin; ●, DHA insulin.

eluate, but this time without heat, showed some biological activity in the rabbit and with the isolated rat diaphragm. These biologically active samples were more completely characterized by DNP *N*-terminal end-group assays and by analysis, and it was shown that not more than 3% of unchanged insulin could be present and that no new free amino groups were produced. The results of the biological assays are shown in Table 2. The DHA insulin showed little activity in the rabbit at a dose of 150 μg. but at 1000 μg. showed activity in different rabbits, which in any individual animal could be matched by a control of 150 μg. of a sample of zinc-free insulin which had also been passed through the silane-treated Celite column and extracted in exactly the same way as was DHA insulin.

The results of the experiments with glucose uptake of the isolated rat diaphragm gave a dose-response curve with DHA insulin that was parallel to that of untreated insulin, showing that the nature and mechanism of their actions were similar (see Fig. 4). It was calculated that 1 μg. of DHA insulin had the same activity as 0·174 μg. of insulin (the 95% confidence range was 0·108–0·279). Since not more than 3% of unchanged insulin could be present in the DHA insulin used in these experiments, the biological activity of 10–28% found above could not be attributed to contamination with unchanged insulin. The interpretation of

such a result would appear to be that the part of insulin corresponding to the heptapeptide is not essential for full biological activity and should not be regarded as the 'active centre' but that it clearly has some influence on the activity.

There was the possibility that a mixture of DHA insulin, the heptapeptide and alanine might give the biological activity of insulin. Table 4 shows that this is not the case, the mixture having no more activity than in the glucose uptake of the rat diaphragm with DHA insulin alone.

Again the possibility that DHA insulin might be more susceptible to denaturation demanded a check that the column procedure had not thus affected its activity. This was done in the experiment in which a known weight of the mixture of trypsin and insulin which had been incubated at 37° was tested without any fractionation. It was found to have lower biological activity than a control mixture which had been kept at 4° (Table 5). This showed that DHA insulin could not possess the same activity as insulin, since if this was so the incubated mixture containing it and unchanged insulin would have had the same activity as the control. This experiment also confirmed that a mixture of DHA insulin, heptapeptide and alanine (which were all in the incubated mixture) did not combine in solution to produce the biological activity of insulin.

Table 2 also shows the results of experiments in which insulin was assayed in the presence of the heptapeptide and of DHA insulin respectively. The insulin activity was unchanged, showing that neither has any inhibiting action on insulin.

Mirsky (1957) showed that the peptides in insulin hydrolysates caused competitive inhibition of insulinase in the liver of the intact rabbit and thus prolongation of insulin action. DHA insulin and the heptapeptide in our experiments, however, did not individually prolong insulin action when injected simultaneously with it into the whole animal. One would have expected prolongation of action if either peptide had depressed insulinase action.

Smith, Hill & Borman (1958) have shown that a considerable number of amino acids can be removed by leucine aminopeptidase from the *N*-terminal end of both chains of insulin without complete inactivation. It appears from our experiments, however, that this was not the case with the *C*-terminal end. The removal of asparagine from this end of the glycyl chain results in considerable inactivation, as does the removal of eight amino acids from the phenylalanyl chain. It is possible that this end of the insulin molecule, especially in the region of the second interchain disulphide linkage, may have a particular importance in the mechanism of the biological activity of the hormone.

Table 4. *Effect of products of trypsin-treated insulin on the glucose uptake of the isolated rat diaphragm*

Experimental details are as given in Table 3. Figures in parentheses show the number of hemidiaphragms used.

Expt. no.	Substrate	Amount (μg.)	Glucose uptake (mean ± S.D.; mg. of glucose/g. of tissue/hr.)	*P* (%)
1	Control	—	1·81 ± 0·30 (4)	—
2	Insulin	0·5	3·41 ± 0·25 (6)	<1 (1 v. 2)
3	DHA insulin	5	3·83 ± 0·25 (6)	<1 (1 v. 3)
4	Heptapeptide	10	2·21 ± 0·25 (6)	>5 (1 v. 4)
5	Alanine	10	1·78 ± 0·30 (4)	>5 (1 v. 5)
6	DHA insulin +heptapeptide +alanine	5 10 10	4·00 ± 0·25 (6)	>5 (3 v. 6)

Table 5. *Effect of trypsin-treated insulin and unincubated control on the glucose uptake of the isolated rat diaphragm*

Experimental details were as given in Table 3 and as described in text. Figures in parentheses show the number of hemidiaphragms used.

Expt. no.	Substrate	Amount (μg.)	Glucose uptake (mean ± S.D.; mg. of glucose/g. of tissue/hr.)	*P* (%)
1	Buffer control	—	1·35 ± 0·34 (6)	—
2	Control digest (unincubated)	0·5	5·23 ± 0·34 (6)	<1 (1 v. 2)
3	Incubated digest	0·5	3·91 ± 0·34 (6)	<1 (1 v. 3); <5 (2 v. 3)

SUMMARY

1. The action of carboxypeptidase on insulin and acetylinsulin has been investigated. The results of previous workers that the removal of *C*-terminal alanine does not affect the biological activity of the insulin molecule, but that removal of *C*-terminal asparagine does so, have been confirmed.

2. The action of trypsin on insulin has also been investigated. The peptides resulting have been purified, characterized and tested for biological activity. The peptide DHA insulin, which is insulin without the eight amino acid residues of the carboxyl end of the phenylalanyl chain, still retains about 15% of the biological activity of the intact insulin molecule.

I wish to thank Professor F. G. Young, F.R.S., and Dr F. Sanger, F.R.S., for much valuable advice and encouragement; Dr L. F. Smith for the gift of acetylinsulin and assistance in the column procedures; Mr T. Hollingsworth and Mr R. G. Carpenter, of the Department of Human Ecology, Cambridge, for advice on statistical analysis and computation.

REFERENCES

Andersen, W. (1954). *Acta chem. scand.* **8**, 359.
Blackburn, S. & Lowther, A. C. (1950). *Biochem. J.* **46**, xxvii.
Butler, J. A. V., Dodds, E. C., Phillips, D. M. P. & Stephen, J. M. L. (1948). *Biochem. J.* **42**, 116.
Butler, J. A. V., Dodds, E. C., Phillips, D. M. P. & Stephen, J. M. L. (1949). *Biochem. J.* **44**, 224.
Fraenkel-Conrat, J. & Fraenkel-Conrat, H. (1950). *Biochim. biophys. Acta*, **5**, 89.
Gerngross, O., Voss, K. & Herfeld, T. (1933). *Ber. dtsch. chem. Ges.* **66**, 435.
Gey, G. O. & Gey, M. K. (1936). *Amer. J. Cancer*, **27**, 45.
Harris, J. I. & Li, C. J. (1952). *J. Amer. chem. Soc.* **74**, 2945.
Howard, G. A. & Martin, A. J. P. (1950). *Biochem. J.* **46**, 532.
Kilby, B. A. & Youatt, G. (1954). *Biochem. J.* **57**, 303.
Lens, J. (1949). *Biochim. biophys. Acta*, **3**, 367.
Levy, A. (1954). *Nature, Lond.*, **174**, 126.
Mirsky, I. A. (1957). *Recent Progr. Hormone Res.* **13**, 429.
Moore, S. & Stein, W. H. (1951). *J. biol. Chem.* **192**, 663.
Nelson, N. (1944). *J. biol. Chem.* **178**, 375.
Nicol, D. S. H. W. & Smith, L. F. (1956). *Biochem. J.* **64**, 17P.
Polson, A., Mosley, V. M. & Wyckoff, R. W. G. (1947). *Science*, **105**, 603.
Randle, P. J. (1956). *J. Endocrin.* **13**, 1.
Ryle, A. P., Sanger, F., Smith, L. F. & Kitai, R. (1955). *Biochem. J.* **60**, 541.
Sanger, F. (1945). *Biochem. J.* **39**, 507.
Sanger, F. & Thompson, E. O. P. (1953). *Biochem. J.* **53**, 366.
Sluyterman, L. A. A. (1955). *Biochim. biophys. Acta*, **17**, 169.
Smith, E. L., Hill, R. L. & Borman, A. (1958). *Biochim. biophys. Acta*, **29**, 207.
Somogyi, M. (1941). *J. biol. Chem.* **141**, 219.
Van Abeele, F. R. & Campbell, E. D. (1951). *Fed. Proc.* **10**, 263.

Biochem. J. (1960) **75**, 401

Glutamic–Aspartic Transaminase of *Dolichos lablab*: Participation by Iron as a Cofactor

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The possibility of a metal participating in enzymic transformations of amino acids that are pyridoxal phosphate-dependent was suggested by the studies of Metzler, Ikawa & Snell (1954). Experimental evidence for metal participation in these types of enzymic reactions was given by Yanofsky (1952), who showed that inhibition of serine dehydrase by metal-binding agents was partially reversed by Mg^{2+} ions. Similar results have been obtained with kynureninase of *Neurospora* (Jakoby & Bonner, 1953), cystathionase of *Escherichia coli* (Wijesundera & Woods, 1953) and tryptophanase (Happold & Struyvenberg, 1954). Eggleston (1958) studied decarboxylases from animal and plant sources and stated that pyridoxal phosphate in some cases acted in conjunction with a metal whereas in others the action was independent of a metal.

Happold & Turner (1957) have shown activation of glutamic acid–aspartic acid transaminase (from heart muscle) by Mg^{2+} ions, but they thought that the effect was due mainly to the acceleration of non-enzymic decarboxylation of oxaloacetic acid, one of the reaction products, thus giving an apparent increase in activity. A preliminary communication from this Institute (Patwardhan, 1958) has

reported the possibility of participation of a metal in transamination catalysed by the enzyme isolated from a plant source, *Dolichos lablab*. The present paper gives more data in support of the idea of iron involvement in the reaction. Purification of the enzyme and some of its properties are also reported.

EXPERIMENTAL

Enzyme. The source of the enzyme was fresh, green beans of *Dolichos lablab*, a seasonal dicotyledonous legume. In a general study of the composition of this material because of its hypoglycaemic effect (Srinivasan, 1957), strong transaminase activity was noticed and this formed the starting-point of the present investigation. The cotyledons were collected from fresh beans by removing the epidermis. An acetone-dried powder of the cotyledons was prepared by homogenizing them, with 3 vol. of distilled acetone cooled to 2°, in a Waring Blendor for 45 sec. and immediately filtering off the acetone on a Büchner funnel. The last traces of acetone were removed from the powder under vacuum. Operations were carried out in a room at 2°. The powder was stored in a desiccator at 2° and was used for the isolation of the enzyme (transaminase); the powder retained its full activity for about 6 months.

Assay of enzyme activity. Transaminase activity of various preparations was determined by the method of Tonhazy, White & Umbreit (1950). Reaction mixtures contained 0·1 ml. of enzyme, 20 μ-moles of DL-aspartic acid, 20 μ-moles of α-oxoglutaric acid and buffer to a volume of 2 ml. Both the substrates were brought to pH 7·4 by addition of dilute alkali. Phosphate buffer (0·081 M-K_2HPO_4–0·019 M-KH_2PO_4), pH 7·4, was first used but later 0·05 M-2 amino-2-hydroxymethylpropane-1:3 diol (tris)–HCl buffer, pH 8·5, was used. Tubes containing buffer, enzyme and aspartic acid were incubated for 5 min. at 37°, after which α-oxoglutaric acid was added. The tubes were immediately placed under nitrogen and incubated for a further 30 min. Enzyme blank consisted of buffer, enzyme and α-oxoglutaric acid. The reaction was stopped by the addition of 0·2 ml. of 10% metaphosphoric acid (Sall, Richards, Harrison & Myerson, 1957). The oxaloacetic acid that was formed as one of the reaction products was then converted into pyruvic acid with aniline citrate and the pyruvate formed was measured as its 2:4-dinitrophenylhydrazone as described by Tonhazy *et al.* (1950), with filter no. 540 in a Klett–Summerson photoelectric colorimeter. Activity of the enzyme is expressed as μg. of pyruvate formed in 30 min./mg. of protein at 37°. When the reaction was studied in the reverse direction, with glutamic acid and oxaloacetic acid as reactants, the aspartic acid formed was first separated with the help of descending chromatograms, with phenol–water (70:30, v/v) in the presence of 3% ammonia in the chamber as the developing solvent. Chromatograms were developed at 23°. Spots were developed with 0·1% ninhydrin in butanol and the colour was measured by extracting it into 75% ethanol, with filter no. 560 in the same instrument as mentioned above. The other product, α-oxoglutaric acid, was estimated as its hydrazone by chromatography. The hydrazone was prepared by the method of Isherwood & Cruickshank (1954). Butanol–ethanol–water (50:10:40, by vol.) was used as the developing solvent. The ascending chromatograms were developed for 18 hr. at 23°. The yellow spots were extracted with 2 ml. of 10% sodium carbonate. The red colour developed after addition of 4 ml. of 2 N-sodium hydroxide was read with filter no. 540 (Tulpule & Patwardhan, 1952).

Protein. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Iron. Iron determinations were carried out by the method of Mahler & Elowe (1954). For this estimation, all the steps involved in enzyme purifications as well as preparation of various reagents were carried out with glass-distilled water. All glassware used for iron determination was Pyrex.

Chemicals. All chemicals used were of reagent grade. DL-Aspartic acid was dissolved in hot water and then precipitated with ethanol. The glutamic acid and aspartic acid employed were found to be chromatographically pure. α-Oxoglutaric acid was precipitated from ethyl acetate by light petroleum. Oxaloacetic acid was kindly supplied by Dr P. S. Sarma.

Solutions of sodium salt of L-thyroxine and of 3:5-diiodotyrosine (British Drug Houses Ltd.) were prepared immediately before use by dissolving the compounds in a minimum amount of alkali and making up to volume with CO_2-free distilled water (Wolff & Wolff, 1957).

Pyridoxal phosphate and bovine serum albumin (Armour and Co.) were kindly supplied by (the late) Dr K. V. Giri. Ethylenediaminetetra-acetic acid (disodium salt) was a gift from Glyco Products, N.Y. For purification studies, Dowex-2 × 8 (20–50 mesh), chloride form, was used. This was washed thoroughly with 6 N- and 3 N-HCl and then with water before use.

Purification of the enzyme

I. *Water extraction.* Acetone-dried powder (1 g.) was extracted with 10 ml. of ice-cold water for 10 min. This and all the subsequent operations were carried out at 2°. The extract was then centrifuged in a refrigerated centrifuge at 2°, at 1000 *g* for 5 min. The supernatant, which was slightly turbid, was collected and the residue discarded.

II. *Treatment with Dowex-2* (Cl *form*). The supernatant was treated with the anion-exchange resin (1 g. of resin for 1 g. of the original powder). After being stirred continuously for 10 min. it was allowed to stand for another 10 min. The resin and the white precipitate that formed were centrifuged off at 1000 *g*, and the supernatant, which was still slightly turbid, was used for the next step. Almost all of the activity of the original extract was recovered after this treatment.

When Dowex-50 in hydrogen form was used, the enzyme extract lost all the activity, although the protein content of the extract was the same as that of the solution treated with anion-exchange resin; it was not possible to restore the activity by the addition of boiled juice of the active enzyme or pyridoxal phosphate or iron, or both the last-named, and no activity was present in the resin eluate. With the anion-exchange resin, similar increases in specific activity of the enzyme were obtained with Dowex-2 in chloride form or with the weak resin IR-4 B.

III. *Treatment with alumina-Cγ.* At this stage the enzyme solution (pH 6·9) contained 37 mg. of total protein. To this solution was added 1 ml. of alumina-Cγ gel (1 ml. of gel equivalent to 90 mg. dry wt.). Before addition of the gel, excess of water from the gel was always centrifuged off to avoid dilution of the enzyme solution. After stirring

Table 1. *Purification of the enzyme*

Reactants: aspartic acid, 20 μmoles; α-oxoglutaric acid, 20 μmoles; enzyme, 0·1 ml.; buffer (0·081 M-K_2HPO_4–0·019 M-KH_2PO_4), pH 7·4; incubation was for 0·5 hr. at 37° under nitrogen. All the operations of purification were carried out at 2°.

Purification stage	Protein (mg./ml.)	Specific activity (μg. of pyruvate/ mg. of protein)	Pyruvate formed (μg.)	Total protein (mg.)
I. Water extract of acetone-dried powder	15·8	86	10 600	123
II. Dowex-2 (Cl form) treatment	5·1	290	10 600	37
III. Supernatant from alumina-Cγ treatment	1·5	616	5 800	9·4
IV. Calcium phosphate gel eluate	0·4	1 420	3 550	2·5
V. Ammonium sulphate fraction between 45 and 75% saturation	0·4	1 525	1 990	1·3
VI. Alumina-Cγ gel eluate	—	2 000	1 494	0·7

with the gel for 10 min. the gel was separated by centrifuging and the clear solution was taken for further treatment.

IV. *Calcium phosphate gel eluate.* Enzyme solution from the step III, which now contained about 10 mg. of protein, was treated with 1 ml. of calcium phosphate gel (1 mg. of gel equivalent to 28 mg. dry wt.). At the end of 15 min. the gel was separated. The supernatant had very little activity. The gel was washed twice with 5 ml. portions of water and then eluted with 5 ml. of 0·1 M-phosphate buffer (K_2HPO_4–KH_2PO_4), pH 7·4.

V. *Treatment with ammonium sulphate.* The eluate (4·2 ml.) was made up to 5 ml. by addition of the last-mentioned buffer and then fractionated with ammonium sulphate at 0°. Solid ammonium sulphate (1·4 g.) was added over a period of 5 min. to bring the solution to 45% saturation. After 15 min. the precipitate formed was removed by centrifuging in the refrigerated centrifuge. The supernatant (5 ml.) was brought to 75% saturation by addition of 1·03 g. of solid ammonium sulphate. After keeping for 30 min., the precipitate formed was collected. This was taken up in 3 ml. of 0·1 M-potassium phosphate buffer, pH 7·4, and dialysed for 2 hr. at 2° against precooled distilled water to remove traces of ammonium sulphate.

VI. *Second treatment with alumina-Cγ gel.* After dialysis, the enzyme solution (protein content 1·3 mg.), pH 7·4, was treated with 0·5 ml. of gel. After 10 min. the solution was centrifuged and the gel eluted with 2 ml. of 0·1 M-potassium phosphate buffer, pH 7·4.

Results of purification are given in Table 1. The enzyme preparation used for most of the studies was purified to stage IV. Attempts to purify the enzyme after stage VI did not meet with any success.

RESULTS

Effect of pH. Behaviour of the enzyme in the presence of various buffers was studied over a range pH 5·0–9·0. The optimum pH of the enzyme was found to lie between 8·2 and 8·5. Cruickshank & Isherwood (1958) have also reported the pH optimum for glutamic acid–aspartic acid transaminase from wheat germ to be between pH 8·0 and 8·5. Lower enzyme activity was obtained when sodium phosphate buffer (Na_2HPO_4–NaH_2PO_4) was used than when the potassium phosphate buffer was employed.

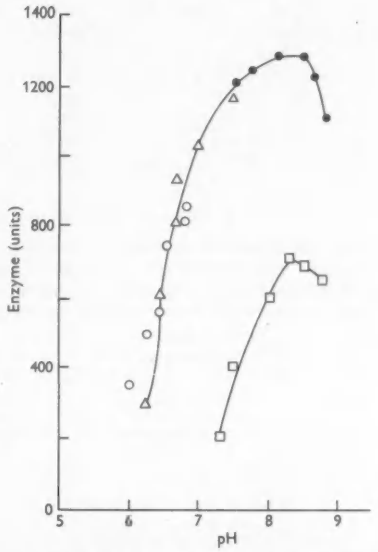

Fig. 1. Effect of various buffers on transaminase. ○, Citric acid–Na_2HPO_4 (0·1 M); △, KH_2PO_4–K_2HPO_4 (0·1 M); ●, 0·05 M-tris–HCl; □, tris–0·05 M-maleic acid–0·05 M-NaOH. Incubation was for 0·5 hr. under nitrogen at 37°.

The enzyme activity over the same range of pH in tris–HCl buffer was significantly higher than with tris–maleic acid–NaOH buffer (Fig. 1). This may have been due either to the presence of Na^+ ions or to the maleic acid. In tris–maleic acid–NaOH buffer, the pH optimum was about 8·2, whereas in tris–HCl buffer it was 8·5.

To find whether this inhibitory effect was due to the presence of maleic acid, the effect of addition of various dicarboxylic acids (oxalic, malonic,

succinic, malic, maleic, fumaric and *o*-phthalic) on the transaminase activity was studied. All the acids were added as their potassium salts with final concentration at 5 mM. It was observed that whereas all the other acids gave 10–12 % inhibition, maleic acid and *o*-phthalic acid gave about 30 % inhibition. As maximum activity was observed with tris–HCl buffer, pH 8·5, this has been employed in most of the studies. It was noticed that over the same range of pH, activity in citrate–phosphate buffer was almost equal to that in phosphate buffer.

Effect of cations on transaminase activity. Preliminary studies indicated that calcium, copper, lithium and zinc did not significantly affect the activity, whereas nickel, manganese and magnesium lowered it considerably. Mercury completely inhibited the activity. Iron in the ferrous state gave a slight increase whereas in the ferric state it was not active. Further studies were therefore continued with ferrous iron. Earlier studies had shown that much activity is lost after dialysis and could be restored to a great extent by the addition of iron as ferrous sulphate (Patwardhan, 1958). Complete restoration could not be obtained. This was suspected to be due to the difference in duration of dialysis of various preparations. It was noticed that when the dialysis was carried out against 8-hydroxyquinoline (5 mM) only for 18 hr., addition of iron restored the activity. After prolonged dialysis (36 hr.) against phosphate buffer, addition of pyridoxal phosphate gave an increase in activity (Table 2). When the enzyme preparation was dialysed, first for 36 hr. against phosphate buffer and then against 8-hydroxyquinoline buffer, it was observed that separate additions of iron and pyridoxal phosphate were not sufficient, but the presence of both was necessary to restore activity (Table 3). This indicates the requirement for both pyridoxal phosphate and iron. It was not possible in the last stage completely to restore the activity to that of the original enzyme.

It is known that metals can catalyse the oxidative decarboxylation of some α-oxodicarboxylic acids (Krebs, 1942; Speck, 1949). Thus it was possible that iron might have decarboxylated non-

Table 2. *Activation of transaminase by pyridoxal phosphate*

The enzyme purified up to stage IV was dialysed for 36 hr. against potassium phosphate buffer, pH 7·4, in a cold room. Reaction buffer: tris–HCl, pH 8·5; incubation was for 0·5 hr. at 37° under nitrogen. Iron was added as $FeSO_4,7H_2O$. Order of additions: enzyme, iron, pyridoxal phosphate, aspartic acid, α-oxoglutaric acid. Amounts of Fe^{2+} ions and pyridoxal phosphate used were 10 μg. each.

Treatment of enzyme	Addition	Units (μg. of pyruvate/ mg. of protein)
1. Dialysed	Nil	285
2. Dialysed	Fe^{2+} ions	450
3. Dialysed	Pyridoxal phosphate	830
4. Dialysed	Fe^{2+} ions + pyridoxal phosphate	900
5. Undialysed	Nil	1200

Table 3. *Effect of pyridoxal phosphate and ferrous iron on transaminase activity*

The enzyme preparation employed in the experiments of Table 2 was, at the end of 36 hr., further dialysed for 18 hr. against aqueous 5 mM-8-hydroxyquinoline in the cold. Reaction buffer: tris–HCl, pH 8·5; incubation was for 0·5 hr. under nitrogen. Order of additions was the same as that given in Table 2. Amounts of Fe^{2+} ions and pyridoxal phosphate used were 10 μg. each.

Treatment of enzyme	Addition	Units (μg. of pyruvate/ mg. of protein)
1. Dialysed	Nil	166
2. Dialysed	Pyridoxal phosphate	261
3. Dialysed	Fe^{2+} ions	309
4. Dialysed	Fe^{2+} ions + pyridoxal phosphate	725

Table 4. *Absence of formation of pyruvate in the enzyme reaction*

Treatment of enzyme	Addition	Units (μg. of pyruvate/ mg. of protein)
1. Dialysed	Nil	400
2. Dialysed	Fe^{2+} ions (10 μg.)	880
3. Dialysed (no aniline citrate was added during colour development)	Fe^{2+} ions (10 μg.)	50
4. Undialysed	Nil	987

enzymically the oxaloacetic acid (one of the reaction products), converting it into pyruvic acid and thus shifting the equilibrium so as to give an apparent increase in the enzyme activity. To test this, an experiment was carried out, the results of which are described in Table 4. It is observed that in the absence of aniline citrate (used in the method of Tonhazy, White & Umbreit, 1950) to convert oxaloacetic acid into pyruvic acid, no pyruvate formation could be demonstrated.

The participation by iron in the enzymic transamination was further substantiated by employing another method. This time, the reaction was studied in the reverse direction. Glutamic acid and oxaloacetic acid were added to the reaction mixture and, at the end of the incubation period, the reaction products aspartic acid and α-oxoglutaric acid were determined. The same effect of iron on the enzymic transamination was noticed. Dialysis of the enzyme decreased the formation of both α-oxoglutaric acid and aspartic acid and addition of iron restored it to a considerable extent (Table 5).

When oxaloacetic acid alone was incubated with the enzyme, it was observed that only about 8% of oxaloacetic acid was converted into pyruvic acid at the end of 30 min. Pyruvic acid thus formed can transaminate with glutamic acid, which is another reactant, to give alanine and thus disturb the equilibrium of the glutamic acid–aspartic acid transaminase reaction. Although the enzyme preparation used in the above-mentioned experiment was purified to stage IV, it had about 15% of glutamic acid–alanine transaminase activity in the presence of added pyruvic acid compared with glutamic acid–aspartic acid transaminase activity. When, however, glutamic acid and oxaloacetic acid were incubated in the presence of enzyme, we could not detect (on paper chromatograms) any alanine formation in the absence of externally added pyruvic acid. Under our experimental conditions any interference due to glutamic acid–pyruvic acid transaminase in our reaction was therefore thought to be small.

Effect of thyroxine on transaminase activity. While this work was in progress, a report appeared indicating that the hormone thyroxine inhibited the various dehydrogenases which require zinc for their activity (Wolff & Wolff, 1957). Lardy (1954) has shown that some bivalent cations, such as Cu^{2+}, Co^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+} and Mg^{2+}, can form complexes with thyroxine under appropriate conditions. In the light of this, we thought that the effect of thyroxine in our present studies might give us more indication of the role of iron in transaminase. The thyroxine effect was studied by pre-incubating the enzyme with thyroxine before the addition of aspartic acid and α-oxoglutaric acid (Table 6). It was observed that 0·1 mM-thyroxine decreased the activity of transaminase to about 75%. When L-thyroxine and iron are added together to the enzyme system so that there is competition for thyroxine between the iron of the enzyme and the externally-added metal, there is less inhibition than in presence of thyroxine only and the inhibition is still further lowered when the

Table 5. *Effect of iron on glutamic acid–aspartic acid transaminase reaction studied in the reverse direction*

Reactants added: glutamic acid 20 μmoles; oxaloacetic acid 20 μmoles; enzyme 0·1 ml.; potassium phosphate buffer (0·1 M), pH 7·4. Incubation was under nitrogen at 37° for 0·5 hr.

Treatment of enzyme	Addition	Aspartic acid formed (μmoles)	α-Oxoglutaric acid formed (μmoles)
1. Dialysed	Nil	5·1	5·4
2. Dialysed	Fe^{2+} ions (20 μg.)	7·7	8·2
3. Undialysed	Nil	9·0	11·2

Table 6. *Effect of thyroxine on transaminase*

L-Thyroxine (sodium salt) was dissolved in a minimum amount of dilute alkali. Iron was added as $FeSO_4,7H_2O$.

Tube no.	Thyroxine (μg.)	Fe^{2+} ions (μg.)	Molar ratio (thyroxine: iron)	Units (μg. of pyruvate/ mg. of protein)
1	Nil	Nil	—	1320
2*	200	Nil	—	543
3†	200	14	1	675
4†	200	28	0·5	806
5‡	200	14	1	950
6‡	200	28	0·5	996

* Thyroxine was added before addition of aspartic acid.
† Thyroxine and iron were added simultaneously.
‡ Thyroxine and iron were pre-incubated for 10 min. and then enzyme and other reactants were introduced.

Table 7. *Iron content and the specific activity of transaminase*

Reactants: aspartic acid 20 μmoles; α-oxoglutaric acid 20 μmoles; enzyme 0·1 ml.; buffer, tris–HCl, pH 8·5; incubation was for 0·5 hr. at 37° under nitrogen.

Treatment	Specific activity (μg. of pyruvate/mg. of protein)	Iron content (μmg./mg. of protein)	Specific activity/iron content
1. Water extract of acetone-dried powder	135	38	3·55
2. Dowex-2 (Cl form) treatment	427	120	3·55
3. Alumina-Cγ supernatant	1300	428	3·05
4. Calcium phosphate gel eluate	1393	440	3·16

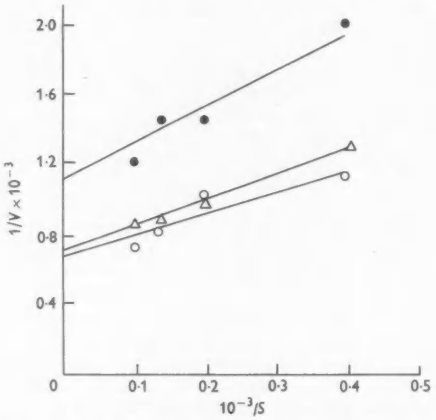

Fig. 2. Inhibition of transaminase by L-thyroxine. ○, Control; △, L-thyroxine (sodium salt), final concentration 25 μmoles; ●, L-thyroxine (sodium salt), final concentration 50 μmoles; aspartic acid and thyroxine were added simultaneously. Buffer, tris–HCl; incubation was for 0·5 hr. under nitrogen at 37°. The lines are drawn by the method of least squares.

amount of iron added is doubled. If thyroxine is pre-incubated with the same amount of iron, and then both are added to the enzyme the inhibition is less and it appears as if thyroxine has been bound by the added iron so that the available thyroxine to react with iron of the enzyme is considerably decreased. We were not able to show any shift in the absorption peak (325 mμ) of thyroxine when the hormone was incubated with the enzyme, although a slight depression in the thyroxine-absorption spectrum was noted. 3:5-Di-iodotyrosine did not have any effect on the activity. When the results are plotted as $1/V$ against $1/S$ it appears that the inhibition is of a non-competitive nature (Fig. 2).

Estimation of iron. Iron estimation was carried out in the presence of hydroxylamine hydrochloride. The results indicate that the iron content increases as purification proceeds; the ratio of iron (μmg.) to specific activity of the enzyme is almost constant during the various stages of purification (Table 7).

Table 8. *Inhibition of transaminase activity by citrate*

Reactants: aspartic acid 20 μmoles; α-oxoglutaric acid 20 μmoles; enzyme purified to stage IV and citrate were pre-incubated in the presence of buffer for 15 min. before addition of substrates. Incubation was under nitrogen at 37° for 0·5 hr.

Buffer	pH	Final concn. of citrate (mM)	Inhibition (%)
1. KH_2PO_4–K_2HPO_4	6·4	5	Nil
2. KH_2PO_4–K_2HPO_4	6·8	5	13
3. KH_2PO_4–K_2HPO_4	7·4	5	40
4. Tris–HCl	7·6	5	33
5. Tris–HCl	8·2	5	40

Effect of metal-binders. The effect of various iron-binders and chelating agents on transaminase activity was studied and a wide range of inhibition was demonstrated by various compounds. The inhibitors were added at a final concentration of 5 mM and the reaction was carried out in potassium phosphate buffer, pH 7·5. The enzyme was pre-incubated with the inhibitor for 10 min. at 37° before the reactants, aspartic acid and α-oxoglutaric acid, were added. In general, chelating agents such as 8-hydroxyquinoline and $\alpha\alpha$-dipyridyl did not show much inhibition whereas chelating agents such as pyrophosphate and citrate gave between 25 and 40% inhibition. Cyanide almost completely destroyed the activity. Next to cyanide, citrate appeared to be the most effective inhibitor. It is known that trisodium and disodium citrate chelate Fe^{2+} ions powerfully to form a complex (Bobtelsky & Jordan, 1945). The complex is stable on the alkaline side of neutrality and breaks down as the acid side is approached. The results presented in Table 8 also indicate that no inhibition by citrate is observed below pH 6·8, but between pH 6·8 and 7·4 the inhibition increased.

No inhibition due to citrate could be observed when the reaction was studied in the range pH 6·0–7·2 with citrate–phosphate and phosphate buffers. The final concentration of citrate in citrate–phosphate buffer varied from 0·01 M at

pH 6·0 to 5 mM at pH 7·2, whereas in the inhibition studies mentioned above, final citrate concentration was 5 mM. This observation of citrate inhibition can thus be taken as additional evidence for participation of iron in the enzymic transamination.

DISCUSSION

This study gives sufficient evidence to suggest the involvement of a metal in enzymic transamination. Participation of pyridoxal phosphate in this reaction has already been established by other workers. In the present study it was possible to remove the metal by dialysis against 8-hydroxyquinoline, when the enzyme activity was reduced and it could be restored to a considerable extent by adding the iron again. This effect of iron has been demonstrated by studying its influence on the formation of three different reaction products, i.e. oxaloacetic acid, aspartic acid and α-oxoglutaric acid, when the reaction was run in the other direction. Chemical analysis for iron indicated that iron accompanied the enzyme through the various stages of purification studied and the ratio of iron (μmg./mg. of protein) to the specific activity was also constant. Thyroxine, which is capable of binding metals (Lardy, 1954), showed inhibition of transaminase activity. These observations indicate that iron is the most likely participant in transamination.

The addition of certain iron-chelating agents to the reaction mixture did not significantly lower the activity of the enzyme, suggesting that the metal bound to the enzyme is not equally accessible to all metal-binders.

Metzler *et al.* (1954) have suggested the mode of participation of metal in this transamination. The first step in transamination is known to be a Schiff's-base formation between the donor amino acid and the pyridoxal phosphate that is already on the enzyme, attached probably through its phosphate group and the ring nitrogen. The presence of metal can be expected to promote the formation of the Schiff's base and the maintenance of planarity of the conjugated system through chelate-ring formation (Metzler *et al.* 1954). The chelated-metal ion also provides an additional electron-attracting group that operates in the same direction as the heterocyclic nitrogen atom, thus increasing the electron displacement from the α-carbon atom. If this is true, it would mean that the role of metal is somewhat secondary to that of pyridoxal phosphate in that the presence of metal helps to initiate or speed the reaction which can potentially take place even in the presence of pyridoxal phosphate alone. It is also possible, as suggested by Williams & Nielands (1954), that combination of the carbonyl group of amino acid with the metal will weaken the base strength of the amino group so that it readily condenses with the formyl group of pyridoxal phosphate.

The present study has given an indication of the role of metal in plant transaminase. It would be interesting if the same effect can also be demonstrated in transaminase from bacterial and animal sources. Quite recently the involvement of iron in another pyridoxal phosphate-dependent enzyme system, namely the biosynthesis of δ-aminolaevulinic acid, has been suggested by Brown (1958), in chicken-erythrocyte preparations.

SUMMARY

1. Glutamic acid–aspartic acid transaminase has been purified about 23-fold from a plant source, beans of *Dolichos lablab*.

2. Iron has been shown to participate as a co-factor with pyridoxal phosphate in the enzymic transamination. Iron estimations were carried out during the various stages of purification and the ratio of iron to specific activity was found to be constant.

3. The participation of iron has been demonstrated by studying its influence on the formation of oxaloacetic acid, α-oxoglutaric acid and aspartic acid respectively when the reaction was studied in both directions.

4. The optimum pH of the enzyme was found to be between 8·2 and 8·5.

5. Thyroxine inhibited the transaminase activity. Thyroxine incubated with iron before addition to the enzyme system inhibited the activity to a considerably less extent than when only thyroxine was added.

I wish to express my thanks to Dr M. Srinivasan, Assistant Director, for helpful discussions and suggestions during the course of this investigation. I am also grateful to Dr V. Subrahmanyan, Director, for his keen interest in the work. Thanks are also due to Glyco Products Co. Inc., U.S.A., for a kind gift of ethylenediaminetetra-acetic acid (disodium salt).

REFERENCES

Bobtelsky, M. & Jordan, J. (1945). *J. Amer. chem. Soc.* **67**, 1824.

Brown, E. G. (1958). *Nature, Lond.*, **182**, 313.

Cruickshank, D. H. & Isherwood, F. A. (1958). *Biochem. J.* **69**, 189.

Eggleston, L. V. (1958). *Biochem. J.* **68**, 557.

Happold, F. C. & Struyvenberg, A. (1954). *Biochem. J.* **58**, 379.

Happold, F. C. & Turner, J. M. (1957). *Nature, Lond.*, **179**, 155.

Isherwood, F. A. & Cruickshank, D. H. (1954). *Nature, Lond.*, **173**, 121.

Jakoby, W. B. & Bonner, D. M. (1953). *J. biol. Chem.* **205**, 699.

Krebs, H. A. (1942). *Biochem. J.* **36**, 303.
Lardy, H. A. (1954). *Brookhaven Symp. Biol.* **7**, 90. Cited in Wolff, J. & Wolff, E. C. (1957), *Biochim. biophys. Acta*, **26**, 387.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
Mahler, H. R. & Elowe, D. G. (1954). *J. biol. Chem.* **210**, 165.
Metzler, D. E., Ikawa, M. & Snell, E. E. (1954). *J. Amer. chem. Soc.* **76**, 648.
Patwardhan, M. V. (1958). *Nature, Lond.*, **181**, 187.
Sall, J., Richards, H. K., Harrison, E. & Myerson, R. M. (1957). *J. Lab. clin. Med.* **50**, 297.
Speck, J. F. (1949). *J. biol. Chem.* **178**, 315.
Srinivasan, M. (1957). *Lancet*, ii, 317.
Tonhazy, N. E., White, N. G. & Umbreit, W. W. (1950). *Arch. Biochem.* **28**, 36.
Tulpule, P. G. & Patwardhan, V. N. (1952). *Nature, Lond.*, **169**, 671.
Wijesundera, S. & Woods, D. D. (1953). *J. gen. Microbiol.* **9**, iii.
Williams, V. R. & Nielands, J. B. (1954). *Arch. Biochem. Biophys.* **53**, 56.
Wolff, J. & Wolff, E. C. (1957). *Biochim. biophys. Acta*, **26**, 387.
Yanofsky, C. (1952). *J. biol. Chem.* **198**, 343.

Biochem. J. (1960) **75**, 408

Regulation of Glucose Uptake by Muscle

4. THE SPECIFICITY OF MONOSACCHARIDE-TRANSPORT SYSTEMS IN RAT-DIAPHRAGM MUSCLE*

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In previous papers (Randle & Smith, 1958*a*, *b*) evidence was presented which showed that the transport of D-glucose and D-xylose across the muscle-cell membrane of isolated rat diaphragm is accelerated by insulin, anoxia and substances which inhibit oxidative phosphorylation (transport is defined as a process in which substances move across the cell membrane in combination with a specific constituent of the cell). These factors were also shown to accelerate transport of D-glucose and L-arabinose in the perfused isolated rat heart (Morgan, Randle & Regen, 1959). The conclusion was drawn by these workers that the transport of sugars across the muscle-cell membrane is inhibited by an energy-rich phosphate compound; that factors such as anoxia, which inhibit oxidative phosphorylation, accelerate transport by lowering intracellular levels of high-energy phosphate compounds; and that insulin accelerates transport by interfering with a reaction between high-energy phosphate and the transport system (Randle & Smith, 1958*a*, *b*).

In the present study the specificity of monosaccharide-transport systems in rat diaphragm has been defined by investigating the extent to which different sugars compete for transport. Evidence has been sought for the nature of the transport system by investigating the effect of some enzyme inhibitors on the transport of sugars. Finally we have attempted to determine whether sugars may pass directly from the transport system into muscle polysaccharides and whether the effect of insulin on sugar transport is secondary to an effect of the hormone on the formation of maltose and glycogen.

* Part 3: Morgan, Randle & Regen (1959).

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METHODS AND PROCEDURE

Incubation and perfusion media. Diaphragms were incubated in the bicarbonate-buffered medium of Gey & Gey (1936) and hearts were perfused with the bicarbonate-buffered medium of Krebs & Henseleit (1932). D-Glucose, D-galactose, D-xylose, D-arabinose, L-arabinose and raffinose were obtained from T. Kerfoot and Co. Ltd., D-mannose, D-fructose and α-methyl-D-glucoside from British Drug Houses Ltd., D-lyxose from California Corporation for Biochemical Research, Los Angeles, U.S.A. and D-3-*O*-methylglucose from Ayerst, McKenna and Harrison, N.Y., U.S.A. These compounds were used without further purification. D-3-*O*-Methylglucose was subjected to chromatography (15 μl. of 1%) on Whatman no. 1 paper with pyridine–ethyl acetate–water (1:2:2) (Jermyn & Isherwood, 1949). Only one substance was detected when the chromatogram was developed with aniline hydrogen phthalate reagent (Partridge, 1949). β-Methyl-D-glucoside was prepared in this Laboratory by Mr B. R. Slater by the Koenig–Knorr reaction.

D-[$^{14}C_6$]Glucose (5 mc/m-mole), D-[1-^{14}C]mannose (1 mc/m-mole) and D-[$^{14}C_6$]sorbitol (6·2 mc/m-mole) were obtained from The Radiochemical Centre, Amersham, Bucks.

Crystalline insulin (Boots Pure Drug Co. Ltd. or Novo Terapeutisk Laboratories, Copenhagen, Denmark) was dissolved in N/300 HCl at a concentration of 20 units/ml. This stock solution was diluted with medium to the required concentration (usually 0·1 unit/ml.) just before each experiment.

Animals. Diaphragm muscle was obtained from male or female albino Wistar rats of 100–150 g. wt. and heart muscle from animals of 200–250 g. wt. The animals were fed with a stock laboratory diet (Bruce & Parkes, 1949) and food was withheld for 18–20 hr. before each experiment. The procedure for removal and incubation of intact diaphragms and hemidiaphragms and for perfusion of heart muscle has been described elsewhere (Randle & Smith, 1958*a*, *b*; Morgan *et al.* 1959).

Analytical methods

Sugars and D-[^{14}C]*sorbitol.* Analyses of muscle for these substances were made on extracts prepared by boiling the muscle in 2–4 ml. of water for 15 min. and deproteinizing with $Zn(OH)_2$ (Somogyi, 1945). This procedure extracts these substances completely. Samples of medium after incubation were suitably diluted and deproteinized in the same way. Pentoses were estimated photometrically by the *p*-bromoaniline method (Roe & Rice, 1948); raffinose was estimated photometrically after hydrolysis and reaction with resorcinol (Roe, Epstein & Goldstein, 1949). Galactose and 3-*O*-methylglucose were estimated photometrically by the Somogyi (1945) modification of Nelson's method, glucose being removed when present by fermentation with baker's yeast. The blank reduction by extracts of muscle under these conditions was so small that it could safely be neglected. [^{14}C]Sorbitol was assayed by methods described elsewhere (Morgan *et al.* 1959).

[^{14}C]*Sugars in muscle.* For identification of [^{14}C]maltose in diaphragm-muscle extracts were prepared and deproteinized as described above. The extract was freed of lipid by ether extraction and desalted by addition of ethanol to 70% (v/v). The extract was dried *in vacuo* and the residue dissolved in 2% maltose + 1% glucose or mannose. Two 5 μl. samples were then chromatographed on Whatman no. 1 paper with either pyridine–ethyl acetate–water (1:2:2) or (where mannose was to be separated from glucose) ethyl acetate–acetic acid–water (3:1:3) (Jermyn & Isherwood, 1949). The chromatogram was developed with aniline hydrogen phthalate reagent and the spots so defined were cut out with a cork borer (internal diam. 2·8 cm.). Radioactivity was assayed directly by placing the disk of paper beneath a thin end-window Geiger–Müller tube. At least 1000 counts were recorded for each sample and replicate determinations under these conditions agreed to within 8%.

Glycogen. Muscle was dissolved in 30% KOH at 60° and glycogen precipitated with the addition of 2% Na_2SO_4 (2:5, v/v) and ethanol to 70% (v/v). The crude glycogen was assayed photometrically with anthrone (Seifter, Dayton, Novic & Muntwyler, 1950).

For assay of radioactivity in glycogen the crude polysaccharide was prepared as described above and purified by reprecipitation (twice). The glycogen was then dissolved in water and transferred to a planchet containing a disk of lens tissue and a drop of a saturated aqueous solution of polyvinyl alcohol. The samples were dried under an infra-red lamp and counted immediately, at least 1000 counts being recorded.

To determine whether [^{14}C]glucose or [^{14}C]mannose could be detected in glycogen the polysaccharide (prepared as described above except that 25% NaOH was used instead of 30% KOH) was hydrolysed with N-HCl at 100° for 3 hr. The hydrolysate was neutralized with N-NaOH (phenol red as internal indicator), desalted by addition of ethanol to 70% (v/v) and dried *in vacuo*. The residue was dissolved in 1% glucose and mannose and two 5 μl. samples were subjected to chromatography (method of assay of radioactivity was as described for [^{14}C]sugars in muscle, above).

Design of experiments

Membrane transport of sugars in diaphragm muscle has been studied by measuring their intracellular accumulation. This has been calculated as the intracellular-sugar space, obtained by subtracting the volume of extracellular water (measured as the sorbitol space) from the sugar space of the tissue (for definition of the concept of space see Randle & Smith, 1958*b*). Intracellular accumulation has also been expressed as the ratio of intracellular- to extracellular-sugar concentration $\times 100$% and calculated from

$$\frac{\text{intracellular-sugar space}}{\text{intracellular-fluid volume}} \times 100\%.$$

In studying competition we have measured the volumes of intracellular water (total water − extracellular water), extracellular water and sugar space in the presence and the absence of the competing sugar. The period of incubation was 30–45 min. and this period was chosen because accumulation of sugars such as D-xylose is not complete in this time (Randle & Smith, 1958*b*). Competition for membrane transport is deemed to have taken place if the second sugar decreases the intracellular accumulation of the first sugar.

Intact diaphragms (weighing 3–4 g.) were incubated in 3–15 ml. of medium. The smaller volume of medium was used in some experiments in order to conserve radioactive sorbitol. Nevertheless, the tissue appeared to be in a satisfactory condition after incubation in such a small volume and furthermore the spaces obtained were similar to those obtained with larger volumes of incubation fluid.

RESULTS

Changes in the volumes of intra- and extra-cellular water

In investigating competition between sugars for membrane transport, D-sorbitol was added to the medium in the control series in place of the second sugar, in order to maintain the total solute concentrations of the medium. However, it became apparent during the course of these investigations that substances such as D-sorbitol which do not penetrate cells do not exert the same effect on the volumes of intra- and extra-cellular water as substances which do penetrate. The results of experiments which demonstrate this are summarized in Table 1. Thus although sorbitol, mannose and urea were added to the extracellular fluid at the same concentration (approx. 100 m-osmoles/kg. of water) their effect on the intracellular-fluid volume was

very different. Sorbitol, to which the cell membrane is impermeable, brought about the largest change in intracellular-water volume, and urea, which enters most rapidly, brought about the least change.

Galactose is a sugar which enters the skeletal-muscle cell in appreciable amounts only in the presence of insulin. Hence it was interesting that the effect of a given galactose concentration upon the intracellular-water volume could be substantially decreased by the addition of insulin.

Effect of other sugars and of D-*sorbitol on the transport of* D-*xylose*

The results of these experiments are recorded in Table 2 and Fig. 1. In the absence of insulin, D-glucose and D-3-*O*-methylglucose at a concentration ratio (to xylose) of 3:1 decreased the intracellular accumulation of xylose. In the presence of the hormone xylose accumulation was diminished by D-glucose (about 30%), D-mannose (about 25%) and D-3-*O*-methylglucose (about 15%). D-Galactose, D-fructose, α-methyl-D-glucoside and maltose did not diminish intracellular accumulation of D-xylose. The effect of D-glucose on the accumulation of D-xylose by the diaphragm at 10, 20, 30 and 40 min. of incubation is shown in Fig. 1. Xylose accumulation was found to be inhibited at all periods of incubation.

Effect of other sugars on the transport of L-*arabinose*

The results of these experiments are shown in Table 3. In the presence of insulin and at a concentration ratio (to arabinose) of 3:1 arabinose accumulation was inhibited by D-glucose (about 60%), D-mannose (about 45%) and D-3-*O*-methylglucose. D-Fructose, D-galactose, α-methyl-D-glucoside and β-methyl-D-glucoside did not inhibit accumulation of arabinose.

Effect of D-*glucose on the transport of* D-*galactose and* D-*arabinose*

The results of these experiments are shown in Tables 4 and 5. In the presence of insulin, glucose at a concentration ratio (to galactose) of 1:1 did not inhibit the accumulation of galactose. The

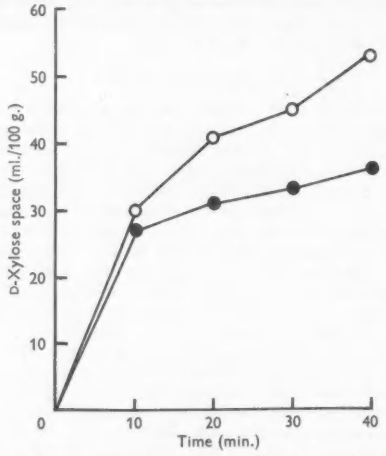

Fig. 1. Effect of D-glucose on the rate of accumulation of D-xylose by the intact diaphragm preparation. Insulin was present at a concentration of 0·1 unit/ml. Each value is the mean of two observations. ○, D-Xylose only (8·3 mM); ●, D-xylose (8·3 mM) + D-glucose (26 mM).

Table 1. *Effect of* D-*sorbitol,* D-*mannose, urea and* D-*galactose on the volumes of total water and extracellular and intracellular water of the intact diaphragm preparation*

Concentration of insulin was 0·1 unit/ml. The volume of extracellular water was measured with D-[$^{14}C_6$]sorbitol. Differences marked with an asterisk are not significant.

Experiment	Concn. (mM)	Insulin	Space (mean ± S.E.M. ml./100 g.) Total water	Extracellular water	Intracellular water	Intracellular water / Total water × 100	No. of observations
Control	—	–	78±0·8	16±1	62±1·7	79·6	6
D-Sorbitol	55	–	75±0·6	23±1·5	52±1·1	69·4	6
Difference			−3±1	7±1·8	−10±2	−10·2	
D-Mannose	100	–	73±0·5	25±0·7	48±1·1	65·6	6
D-Sorbitol	100	–	75±0·4	32±1·1	43±1·7	57·3	6
Difference			2±0·7	7±1·3	−5±2	−8·3	
Urea	100	–	77±0·7	22±2·2	55±2	71·5	6
D-Sorbitol	100	–	75±0·3	35±1·2	40±1·2	53·3	6
Difference			−2±0·8	13±2·5	−15±2·3	−18·2	
D-Galactose	26	–	78±0·9	27±2·7	51±2·2	65·3	4
D-Galactose	26	+	78±0·9	18±0·7	60±0·9	77·1	4
Difference			0±1·3*	−9±2·8	9±2·3	11·8	

Table 2. *Effect of other sugars and of* D-*sorbitol on the accumulation of* D-*xylose by the intact diaphragm preparation*

Differences given are deviations from control values given at the head of the table in parentheses. Concentration of insulin was 0·1 unit/ml. D-[$^{14}C_6$]Sorbitol (0·12 μc/ml.) was added to all media. ICXC, ECXC: intra- and extra-cellular xylose concentrations. Differences marked with an asterisk are not significant ($P > 0{\cdot}05$).

Present in addition to D-xylose (8·3 mM)	Insulin	Difference from control (D-xylose, 8·3 mM + D-[^{12}C]sorbitol, 26 mM) (mean ± S.E.M.; Δ ml./100 g. of wet muscle) Extracellular water (23)	Intracellular water	Xylose space (30)	Intracellular xylose space (7)	ICXC/ECXC (%)	No. of observations
	−		.			.	.
	+	(20)	(55)	(44)	(24)	(44)	.
D-Glucose (26 mM)	−	−5±1·2	.	−8±1·4	−3±1	.	12
D-Glucose (26 mM)	+	−6±1·1	5±1	−12±1·3	−6±1·7	−13±4	12
D-Mannose (26 mM)	+	−2±1·4*	0±1·4*	−9±1·4	−7±1·3	−11±1·8	6
D-3-*O*-Methylglucose (26 mM)	−	−2±0·9*	1±0·8*	−5±1·4	−2±1·0*	−4±1·5	6
D-3-*O*-Methylglucose (26 mM)	+	−3±0·7	3±0·7	−6±0·8	−3±1·1	−7±1·8	16
D-Galactose (26 mM)	+	−4±1·2	6±2·3	−3±1·2	1±1·3*	8±1·9	12
D-Fructose (26 mM)	+	.	.	−1±3·1*	.	.	4
D-Sorbitol (55 mM)†	+	7±1·8	−10±2	3±2·3*	−3±2·8*	0±4·4*	6
α-Methyl-D-glucoside (26 mM)	+	−2±1·7*	.	−2±2·4*	0±1·4*	.	5
Maltose (26 mM)	+	−2±1·7*	.	−1±3*	1±1·4*	.	5

† In this experiment the control contained only D-xylose.

Table 3. *Effect of other sugars on the accumulation of* L-*arabinose by the intact diaphragm preparation*

Differences given are deviations from control values given in parentheses at the head of the table. Concentration of insulin was 0·1 unit/ml. D-[$^{14}C_6$]-Sorbitol (0·12 μc/ml.) was added to all media. ICAC, ECAC: intra- and extra-cellular arabinose concentrations. Differences marked with an asterisk are not significant ($P > 0{\cdot}05$).

Present in addition to L-arabinose (8·3 mM)	Insulin	Difference from control (L-arabinose, 8·3 mM + D-[^{12}C]sorbitol, 26 mM) (mean ± S.E.M.; Δ ml./100 g. of wet muscle) Extracellular water (23)	Intracellular water (54)	Arabinose space (44)	Intracellular arabinose space (21)	ICAC/ECAC (%) (39)	No. of observations
D-Glucose (26 mM)	+	1±0·8*	−1±0·8*	−13±1·8	−12±2	−23±2	4
D-Mannose (26 mM)	+	−1±2·0*	.	−11±1·9	−11±1·9	.	4
D-Galactose (26 mM)	+	−4±2·1*	5±1·8	−1±2·7*	3±3·4*	2±3·4	5
D-3-*O*-Methylglucose (26 mM)	+	.	.	−12±1·7	.	.	5
D-Fructose (26 mM)	+	.	.	−3±2·2*	.	.	5
α-Methyl-D-glucoside (26 mM)	+	−1±0·8*	.	1±2·6*	2±2·3*	.	.
β-Methyl-D-glucoside (26 mM)	+	3±1·6*	.	2±1·5*	−1±2·4*	.	5

Table 4. *Effect of* D-*glucose on the accumulation of* D-*galactose by the intact diaphragm preparation*

Differences given are deviations from control values given in parentheses at the head of the table. Concentration of insulin was 0·1 unit/ml. D-[$^{14}C_6$]-Sorbitol (0·12 μc/ml.) was added to all media. ICGC, ECGC: intra- and extra-cellular galactose concentrations. Differences marked with an asterisk are not significant ($P > 0.05$).

Present in addition to D-galactose (14 mM)	Insulin	Difference from control (D-galactose, 14 mM + D-[^{12}C]sorbitol, 5·5 mM) (mean ± S.E.M.; Δ ml./100 g. of wet muscle)				ICGC/ECGC (%)	No. of observations
		Extracellular water (24)	Intracellular water (51)	Galactose space (54)	Intracellular galactose space (30)	(60)	
D-Glucose (14 mM)	+	−6 ± 1·9	4 ± 2*	2 ± 4·7*	8 ± 5·6*	9 ± 4	6

Table 5. *Effect of* D-*glucose on the accumulation of* D-*arabinose by the intact diaphragm preparation*

Differences given are deviations from control values given in parentheses at the head of the table. Concentration of insulin was 0·1 unit/ml. D-[$^{14}C_6$]-Sorbitol (0·12 μc/ml.) was added to all media. ICAC, ECAC: intra- and extra-cellular arabinose concentration. Differences marked with an asterisk are not significant ($P > 0.05$).

Present in addition to D-arabinose (8·3 mM)	Insulin	Difference from control (D-arabinose, 8·3 mM + D-[^{12}C]sorbitol, 26 mM) (mean ± S.E.M.; Δ ml./100 g. of wet muscle)				ICAC/ECAC (%)	No. of observations
		Extracellular water (21)	Intracellular water (55)	Arabinose space (31)	Intracellular arabinose space (10)	(18)	
D-Glucose (26 mM)	+	−4 ± 1·4	3 ± 1·2	−7 ± 2·1	−3 ± 1·1	−5 ± 2·2	12

Table 6. *Effect of phlorrhizin,* N*-ethylmaleimide,* p*-chloromercuribenzoate and diisopropyl phosphorofluoridate on the accumulation of* D-*xylose and* D-*galactose by the intact diaphragm preparation*

Concentration of insulin was 0·1 unit/ml. Extracellular water was measured with D-[$^{14}C_6$]sorbitol (0·12 μc/ml.) in Expts. 1, 4 and 5 and separately with raffinose (20 mM) in Expts. 2 and 3. ICSC, ECSC: intra- and extra-cellular xylose or galactose concentrations. Differences marked with an asterisk are not significant ($P > 0.05$).

Expt. no.	Present in addition to D-xylose (8·3 mM, Expts. 1–4) or D-galactose (26 mM, Expt. 5)	Insulin	Difference from control (xylose or galactose alone) (mean ± S.E.M.; Δ ml./100 g. of wet muscle)				ICSC/ECSC (%)	No. of observations
			Extracellular water	Intracellular water	Xylose or galactose space	Intracellular xylose or galactose space		
1	Phlorrhizin (3 mM)	+	−1 ± 1·6*	1 ± 1·3*	−15 ± 3·8	−14 ± 3	−23 ± 3	6
2	*N*-Ethylmaleimide (mM)	+	12 ± 1·3	−10 ± 1·7	−8 ± 1·1	−20 ± 1·7	−35 ± 2·2	6
3	*p*-Chloromercuribenzoate (10 μM)	+	−1 ± 0·8*	3 ± 1	−5 ± 1	−4 ± 1·3	−9 ± 2	6
4	Diisopropyl phosphorofluoridate (20 mM)	+	2 ± 1·8*		1 ± 3·3	−1 ± 3·1*		6
5	Phlorrhizin (3 mM)	+	3 ± 1·4	−3 ± 1·8*	−9 ± 3·5	−12 ± 3·5	−20 ± 6·3	6

accumulation of D-arabinose (glucose:arabinose concentration ratio 3:1) was inhibited (about 30%) by glucose.

Effect of D-3-O-*methylglucose on the transport of* D-*lyxose*

The results of this experiment are recorded in Fig. 2. D-3-*O*-Methylglucose at a concentration ratio (to D-lyxose) of 3:1 inhibited lyxose accumulation at 10, 20, 30 and 40 min. of incubation.

Effect of phlorrhizin, N-ethylmaleimide, p-chloromercuribenzoate and diisopropyl phosphorofluoridate on the transport of D-*xylose and* D-*galactose*

The results of these experiments are shown in Table 6. Phlorrhizin (3 mM), *N*-ethylmaleimide (mM) and *p*-chloromercuribenzoate (10 μM) inhibited accumulation of xylose (about 50, 80 and 20% respectively). Di*iso*propyl phosphorofluoridate (20 μM) did not affect the accumulation of xylose. These experiments were made in the presence of insulin. Phlorrhizin (3 mM) also diminished the accumulation of D-galactose in the presence of insulin by about 40%.

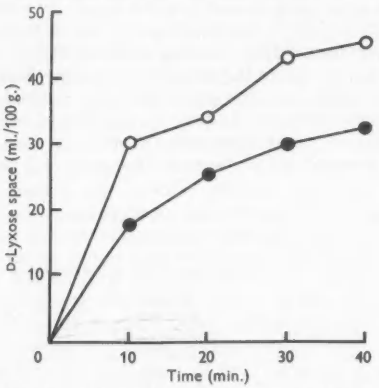

Fig. 2. Effect of D-3-*O*-methylglucose on the rate of accumulation of D-lyxose by the intact diaphragm preparation. Insulin was present at a concentration of 0·1 unit/ml. Each value is the mean of two observations. ○, D-Lyxose only (8·3 mM); ●, D-xylose (8·3 mM) + D-3-*O*-methylglucose (26 mM).

Effect of glucose on the uptake and on the incorporation of ^{14}C *from* [^{14}C]*mannose into glycogen and maltose in the diaphragm*

Since D-mannose is metabolized by diaphragm muscle the extent to which it accumulates in the muscle does not provide a measure of its transport. The uptake of the sugar and its incorporation into glycogen and maltose in isolated diaphragm have therefore been chosen as parameters of the rate of transport. The reasons for accepting rates of utilization as a measure of the rate of transport have been summarized by Randle & Smith (1958*a*).

Beloff-Chain *et al.* (1955) first showed that ^{14}C from [^{14}C]glucose is incorporated into maltose in isolated rat diaphragm and that the extent of this incorporation is enhanced by insulin. This we have confirmed in experiments in which eight hemidiaphragms were incubated for 10 min. at 37° in 3 ml. of bicarbonate medium containing [^{12}C]-glucose (5·75 mg./ml.) and [^{14}C$_6$] glucose (3 μc/ml.). Maltose was isolated from the muscle by chromatography (see Methods and Procedure section) and the radioactivity in maltose was 9400 counts/min./g. of wet muscle/hr. in the presence of insulin and 3520 in the absence of the hormone (an increase of 2·7-fold). The radioactivity in glucose was 26 200 in the presence and 24 500 in the absence of the hormone.

The results of experiments with [^{14}C]mannose are shown in Table 7. The uptake of [^{14}C]mannose from the medium by diaphragm was increased by insulin (2·8-fold) but depressed by [^{12}C]glucose (44%; ratio of concentrations glucose:mannose, 10:1). The incorporation of ^{14}C from [^{14}C]mannose into glycogen and maltose in the presence of insulin was depressed by [^{12}C]glucose (in glycogen 41%, in maltose 49%). Analysis of glycogen

Table 7. *Effect of insulin* (0·1 *unit/ml.*) *and of* [^{12}C]*glucose* (3 *mg./ml.*) *on the uptake and incorporation into maltose and glycogen in isolated rat diaphragm of* ^{14}C *from* D-[1-^{14}C]*mannose* (1·3 μc/*ml.*) *and* D-[^{12}C]*mannose* (0·3 *mg./ml.*)

Measurements were made on four hemidiaphragms incubated together in 3 ml. of medium for 20 min. at 37°

Addition	Insulin	Radioactivity (counts/min./g. of wet diaphragm/hr. of incubation)		
		Uptake of mannose	In glycogen	In maltose
Mannose	−	273 800	.	.
	+	767 000	.	.
Mannose + glucose	+	430 140	.	.
Mannose	+	.	486 714	1880
Mannose + glucose	+	.	288 267	956

formed during incubation with [^{14}C]mannose showed that all the radioactivity was present as glucose residues and no radioactive mannose could be detected in a hydrolysate.

Effect of insulin on glycogen breakdown in diaphragm and on the incorporation of ^{14}C from [^{14}C]glucose into glycogen of rat heart under anaerobic conditions

The effect of insulin on glycogen breakdown in diaphragm muscle was investigated by incubating paired hemidiaphragms with and without insulin under aerobic conditions in medium free of glucose for 1 hr. at 37°. The final concentrations of glycogen (mg. of glycogen glucose/g. of wet diaphragm) were: without insulin, $1{\cdot}7 + 0{\cdot}25$, and with insulin, $1{\cdot}7 \pm 0{\cdot}25$.

The effect of insulin on the incorporation of ^{14}C from [^{14}C]glucose into the glycogen of isolated rat heart in a 15 min. period of perfusion under anaerobic conditions is shown in Table 8. Under these conditions insulin did not increase the incorporation of ^{14}C into glycogen at either of the concentrations of glucose employed and indeed insulin appeared to effect a small reduction, although the differences observed were not significant. On the basis of earlier experiments (Morgan *et al.* 1959) the uptake of glucose under these conditions would be about 4·5 mg. in the presence of insulin and about 2·5 mg. in the absence of the hormone. The volume of perfusate in the present experiments was 10 ml., so that in the presence of insulin almost all of the glucose would be utilized at the lower concentration (0·5 mg./ml.) and about 25 % utilized at the higher concentration (2·0 mg./ml.).

DISCUSSION

The results of these studies of competition are summarized together with the structural features of the sugars in Table 9. We conclude from them that there is more than one monosaccharide-transport system in isolated diaphragm; that one system transports the competing sugars D-glucose, D-mannose, D-3-*O*-methylglucose, D-xylose, D-arabinose, D-lyxose and L-arabinose and that the sugars which do not compete with members of this group (D-galactose and D-fructose) are transported by different systems. α-Methyl-D-glucoside, β-methyl-D-glucose, maltose and D-sorbitol neither compete with D-xylose or L-arabinose, or both, for transport nor do they (unlike the sugars listed above) enter the muscle cell in the presence of insulin. Kipnis & Cori (1959) in similar studies with isolated diaphragm were able to detect competition between D-glucose, D-mannose and D-2-deoxyglucose, but not between D-glucose and D-xylose or D-2-deoxy-

Table 8. *Effect of insulin (0·1 unit/ml.) on incorporation of ^{14}C from D-[$^{14}C_6$]glucose into glycogen of perfused isolated rat heart under anaerobic conditions*

Differences marked with an asterisk are not significant. Numbers of observations are given in parentheses.

Concn.				
[^{12}C]Glucose (mg./ml.)	[^{14}C]Glucose (μc/ml.)	Insulin	Radioactivity (mean ± S.E.M.)	Counts/min. in glycogen/g. of wet heart/hr. of incubation (mean difference ± S.E.M.)
0·5	0·1	−	184 ± 25 (7)	−61 ± 35*
		+	123 ± 25 (7)	
2	0·4	−	1810 ± 41 (2)	−155 ± 160*
		+	1655 ± 156 (2)	

Table 9. *Configuration of sugars and related compounds which do or do not compete for transport in isolated diaphragm*

Sugar or related compound	Competition demonstrated with	No competition with	Position of −OH group above (+) or below (−) plane of pyranose ring				
			C-2	C-3	C-4	C-5	C-6
1. D-Glucose	.	.	−	+	−	.	+
2. D-Mannose	1	.	+	+	−	.	+
3. D-3-*O*-Methylglucose	.	.	−	+(O.CH_3)	−	.	+
4. D-Xylose	1, 2, 3	8, 9, 10, 12, 13	−	+	−	.	.
5. D-Arabinose	1	.	+	−	−	.	.
6. D-Lyxose	3	.	+	+	−	.	.
7. L-Arabinose	1, 2, 3	8, 9, 10, 11	−	+	+	.	.
8. D-Fructose	.	.	±	+	−	−	.
9. D-Galactose	.	1	−	+	+	.	+
10. α-Methyl-D-glucoside	.	.	−	+	−	.	+
11. β-Methyl-D-glucoside	.	.	−	+	−	.	+
12. D-Sorbitol	.	.	.	.	.	.	.
13. Maltose	.	.	.	.	.	.	.

glucose and D-galactose and D-fructose. They conclude that there may be more than one monosaccharide-transport system in diaphragm muscle. Although our observations confirm this suggestion we are unable to confirm that there is no competition between glucose and xylose. Our findings in diaphragm in this connexion are in agreement with the earlier observations of Park, Reinwein, Henderson, Cadenas & Morgan (1959), who have made similar studies in the perfused isolated heart.

In the present study all the monosaccharides which are transported by the same system as glucose are aldose sugars; the only ketose tested (fructose) is not transported by this system. Since pentoses are transported by the same system as hexoses the presence of a methoxy group on C-5 of the pyranose ring is not determinant. Our observations with 3-methylglucose and those of Kipnis & Cori with 2-deoxyglucose show that a hydroxyl group on C-2 or C-3 of the pyranose ring is not essential. Furthermore, our findings show also that the position of hydroxyl groups on C-2, C-3, C-4 or C-6 in relation to the plane of the pyranose ring is not determinant. On the other hand, the lack of competition between α-methyl-D-glucoside or β-methyl-D-glucoside and D-xylose or L-arabinose suggests that a hydroxyl group on C-1 of aldose sugars is essential; an alternative possibility is that a methyl group in this position interferes with transport. D-Galactose, which is not transported by the same system as glucose and other members of this group, differs only in having hydroxyl groups on C-4 and C-6 on the same side of the plane of the pyranose ring. Galactose is unique amongst sugars tested in this respect and it is important to note that L-arabinose, which has hydroxyl groups on the same side of the plane of the pyranose ring as galactose, on C-2, C-3 and C-4, is transported not by the system which transports galactose but by that which transports glucose. We conclude that a free hydroxyl group on C-1 of aldose sugars is necessary for transport, that aldose sugars are transported by a different system from the ketose sugar D-fructose and that the relative positions of hydroxyl groups on C-4 and C-6 in relation to the plane of the pyranose ring may determine whether an aldopyranose is transported by one system or another.

The transport of D-xylose and D-galactose in diaphragm was substantially reduced by phlorrhizin and this is in keeping with effects of phlorrhizin on sugar transport in other mammalian cells and tissues. Keller & Lotspeich (1959) find that phlorrhizin inhibits oxidative phosphorylation in mitochondrial preparations from rat kidney and liver. In view of this our finding that phlorrhizin inhibits sugar transport in diaphragm might appear anomalous, because it has been shown previously that substances such as salicylate or 2:4-dinitrophenol which inhibit oxidative phosphorylation accelerate transport of sugars in diaphragm (Randle & Smith, 1958*b*). We attribute this apparently anomalous effect of phlorrhizin to its inability to penetrate the muscle cell significantly. Thus the phlorrhizin space of isolated diaphragm estimated by the method of Jenner & Smyth (1959) was found to be very similar to the extracellular-fluid volume measured with sorbitol (phlorrhizin space 28 ml./100 g.; sorbitol space 24 ml./100 g.). Furthermore phlorrhizin does not (as would be expected if it penetrated muscle cells and inhibited oxidative phosphorylation therein) induce rigor in isolated diaphragm or alter the contractile activity of perfused isolated rat heart.

The transport of D-xylose was also inhibited by the –SH-group poisons *p*-chloromercuribenzoate and *N*-ethylmaleimide (cf. LeFevre, 1948), but not by di*iso*propyl phosphorofluoridate (thus excluding participation of enzymes or amino acid sequences reacting with this substance; for review see Dixon & Webb, 1958). This would suggest that the –SH group is involved in transport in some way, and if so there appear to be three possibilities in this connexion; (i) that transport involves reaction between the –SH group (possibly on a protein) and a group on a sugar, possibly the –OH group on C-1 of aldose sugars; (ii) that transport involves reaction between a –SH compound and a monosaccharide but that the –SH group does not participate directly in the reaction; (iii) that transport involves reaction between a monosaccharide and a cell constituent under the influence of a –SH enzyme. The third possibility is perhaps less likely than the other two because of the very low specificity of the transport system for D-xylose. Whichever of these explanations is the correct one, it is clear that transport involves reaction of more than one group on monosaccharides because both a free hydroxyl group on C-1 and a particular configuration of hydroxyl groups on C-4 and C-6 appear to be of importance in relation to the transport system for D-xylose.

Beloff-Chain *et al.* (1955) have emphasized that one of the major effects of insulin on carbohydrate metabolism in diaphragm is an enhanced formation of glycogen and of oligosaccharides such as maltose from [^{14}C]glucose. This we have confirmed. They have concluded from their observations that this is a primary site of action of the hormone and that the synthesis of glycogen and oligosaccharides may not involve phosphorylation of glucose by hexokinase. It is to be inferred from these conclusions that an effect of the hormone on sugar transport would be secondary to an effect on the synthesis of glycogen and oligosaccharides. Our findings do not support this view. Thus although insulin approximately doubles uptake of glucose by the perfused

isolated rat heart under anaerobic conditions (Morgan *et al.* 1959) it does not enhance incorporation of glucose into glycogen under these conditions. This would suggest that effects of the hormone on glucose transport are not dependent upon an increased rate of glycogen synthesis. Moreover, if insulin accelerated the formation of glycogen from some intermediate such as glucose 1-phosphate the hormone might also be expected to impede glycogen breakdown by increasing the rate at which glucose 1-phosphate derived from the breakdown of glycogen is incorporated again into the polysaccharide. Insulin did not impede glycogen breakdown in our experiments. Furthermore if the rate of glycogen synthesis in diaphragm does not depend upon transport or proceed through the hexokinase reaction it is difficult to see why glucose should inhibit the incorporation of mannose into glycogen glucose. The reactions involved in the transport and phosphorylation of glucose and mannose are the only points at which competition for entry into glycogen can be visualized as taking place at the present time. We have considered the possibility that glucose and mannose might be transferred from the transport form into glycogen without phosphorylation by hexokinase but this appears to be excluded by our finding that ^{14}C from [^{14}C]-mannose is found in glycogen only as glucose residues. Our observations are in every way consistent with the view first put forward by Levine & Goldstein (1955) and Park, Bornstein & Post (1955) that the rate-limiting step in the uptake of glucose by muscle is transport of the sugar across the cell membrane and that the major effect of insulin is to accelerate transport of the sugar.

SUMMARY

1. The specificity of monosaccharide transport in isolated diaphragm has been investigated by measuring the effect of other sugars on the intracellular accumulations of D-xylose, D-arabinose, D-lyxose, D-galactose and L-arabinose.

2. Competition for transport could be demonstrated between a group of sugars comprising D-glucose, D-mannose, D-3-*O*-methylglucose, D-xylose, D-arabinose, D-lyxose and L-arabinose, but not between members of this group and D-galactose, D-fructose, maltose, α-methyl-D-glucoside, β-methyl-D-glucoside or D-sorbitol.

3. Transport of D-xylose was inhibited by phlorrhizin and the –SH poisons *p*-chloromercuribenzoate and *N*-ethylmaleimide.

4. We conclude that there is more than one monosaccharide-transport system in muscle and that a –SH compound, possibly a –SH protein, is involved in monosaccharide transport. The factors of importance to the specificity of monosaccharide transport in diaphragm are discussed.

5. Insulin did not increase the incorporation of ^{14}C from [^{14}C]glucose into glycogen of perfused isolated rat heart under anaerobic conditions although it increases glucose uptake under these conditions. We conclude that an effect of the hormone on transport of glucose does not depend upon increased formation of glycogen.

6. [^{12}C]Glucose was found to decrease the incorporation of ^{14}C from [^{14}C]mannose into maltose and glycogen in isolated rat diaphragm. ^{14}C from [^{14}C]mannose was present only in glucose residues in glycogen; no [^{14}C]mannose could be detected in the polysaccharide. We conclude that the formation of glycogen from mannose follows transport and phosphorylation of the sugar and that sugars do not pass directly from the transport form into polysaccharide.

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REFERENCES

Beloff-Chain, A., Catamzarro, R., Beloff-Chain, E., Masi, I., Pocchiari, F. & Rossi, C. (1955). *Proc. Roy. Soc.* B, **143**, 481.

Bruce, H. M. & Parkes, A. S. (1949). *J. Hyg., Camb.*, **47**, 201.

Dixon, M. & Webb, E. C. (1958). *Enzymes.* London: Longmans, Green and Co.

Gey, G. O. & Gey, M. K. (1936). *Amer. J. Cancer*, **27**, 45.

Jenner, F. A. & Smyth, D. H. (1959). *Biochem. J.* **71**, 138.

Jermyn, M. A. & Isherwood, F. A. (1949). *Biochem. J.* **44**, 402.

Keller, D. M. & Lotspeich, W. D. (1959). *J. biol. Chem.* **234**, 987.

Kipnis, D. M. & Cori, C. F. (1959). *J. biol. Chem.* **234**, 171.

Krebs, H. A. & Henseleit, K. (1932). *Hoppe-Seyl. Z.* **210**, 33.

LeFevre, P. G. (1948). *J. gen. Physiol.* **31**, 505.

Levine, R. & Goldstein, M. S. (1955). *Recent Progr. Hormone Res.* **11**, 343.

Morgan, H. E., Randle, P. J. & Regen, D. M. (1959). *Biochem. J.* **73**, 573.

Park, C. R., Bornstein, J. & Post, R. L. (1955). *Amer. J. Physiol.* **182**, 12.

Park, C. R., Reinwein, D., Henderson, M. J., Cadenas, E. & Morgan, H. E. (1959). *Amer. J. Med.* **26**, 674.

Partridge, S. M. (1949). *Nature, Lond.*, **164**, 443.

Randle, P. J. & Smith, G. H. (1958*a*). *Biochem. J.* **70**, 490.

Randle, P. J. & Smith, G. H. (1958*b*). *Biochem. J.* **70**, 501.

Roe, J. H., Epstein, J. H. & Goldstein, N. P. (1949). *J. biol. Chem.* **178**, 839.

Roe, J. H. & Rice, E. W. (1948). *J. biol. Chem.* **173**, 507.

Seifter, S., Dayton, S., Novic, B. & Muntwyler, E. (1950). *Arch. Biochem.* **25**, 191.

Somogyi, M. (1945). *J. biol. Chem.* **160**, 69.